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(54) Title: AN ENZYME WITH AMINOPEPTIDASE ACTIVITY

(57) Abstract

The invention relates to a 35 kDa enzyme exhibiting aminopeptidase activity which is derived from a fungal microorganism, a DNA construct comprising a DNA sequence encoding said enzyme, a recombinant expression vector comprising said DNA construct, and a cell comprising said DNA sequence. It is also an object of the invention to provide a method for producing said enzyme exhibiting aminopeptidase activity, and an enzyme preparation comprising said enzyme, a bread-improving or dough-improving composition comprising the aminopeptidase of the invention. Finally the invention relates to the use of said enzyme exhibiting aminopeptidase activity or enzyme preparations or compositions thereof.

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Title: An enzyme with aminopeptidase activity

FIELD OF THE INVENTION

The present invention relates to an enzyme exhibiting aminopeptidase activity, a method for producing said enzyme, an enzyme preparation containing said enzyme exhibiting aminopeptidase activity, and use of said enzyme for various industrial purposes.

BACKGROUND OF THE INVENTION

Protein hydrolysates are being used in numerous food products. Traditionally protein hydrolysates were produced by acid hydrolysis, but today enzymatic hydrolysis is regarded as an attractive alternative.

One of the main problems of protein hydrolysates is that they often taste bitter. When using *e.g.* soy protein or casein, which are rich in hydrophobic L-amino acids, as the protein source, the protein hydrolysate tends to have bitter taste. In general it is believed that whether the taste of proteins is bitter or not depends on the average hydrophobicity of the L-amino acid residues, such as valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan.

A vast number of enzymes exhibiting peptidase activity are capable of performing enzymatic hydrolysis on vegetable, yeast and/or animal proteins, resulting in highly nutritious protein hydrolysates useful as food additives in products such as soups, sauces, gravies, paste, tofu, bouillon, seasonings, baby formulas, snacks, ready-to-eat meals etc.

Peptidases

All peptidases or proteases are hydrolases which act on proteins or its partial hydrolysate to decompose the peptide bond.

EP 427,385 (The Japanese R & D Association) discloses a genomic gene encoding an alkaline protease derived from yellow molds such as *Aspergillus oryzae*.

JP-0-2002374 and JP-0-2002375 (Shokuhin), describes an alkaline protease derived from *Aspergillus oryzae* for use in medicine, food, and detergents.

SU-891777 (Khark) concerns a microbial protease from *Aspergillus oryzae*, which can be used in food, medicine etc.

JP-5-4035283 (Ajinomoto KK) discloses preparation of enzymes from *e.g. Aspergillus oryzae* exhibiting endopeptidase activity, which can hydrolyse proteins almost completely.

WO 94/25580 (Novo Nordisk A/S) describes a method for hydrolysing vegetable or animal protein by incubating with a proteolytic enzyme preparation derived from a strain of *Aspergillus oryzae*.

Aminopeptidases

A subgroup of peptidases (proteases) are called aminopeptidases and are classified under the Enzyme Classification number E.C. 3.4.11 (aminopeptidases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB)).

Aminopeptidases are capable of removing one or more amino terminal residues from polypeptides.

JP-7-5034631 (Noda) discloses a leucine aminopeptidase derived from yellow koji mold, which includes *Aspergillus oryzae*.

JP-7-4021798 (Zaidan Hojin Noda Sangyo) describes the production of miso by adding of a leucine aminopeptidase II prepared by cultivating a number of molds, including *Aspergillus oryzae* strain 460 and strain IAM 2616.

Van Heeke et al., Bioch. Biophys. Acta, (1992), **1131**, 337-340, have disclosed the cloning of a 30 kDa aminopeptidase from the bacteria *Vibrio proteolyticus* deposited at the American Type Culture Collection under the ATCC No. 15338.

Aspergillus oryzae 460 is known to produce a number of leucine aminopeptidases. The molecular weight of three of these was calculated to 26,500, 56,000 and 61,000, respectively determined by gel filtration (Nakada et al., Agr. Biol. Chem, (1972), **37**(4), 757-765; Nakada et al., Agr. Biol. Chem, (1972), **37**(4), 767-774; Nakada et al., Agr. Biol. Chem, (1972), **37**(4), 775-782). The *Aspergillus oryzae* 460 strain is deposited at the American Type Culture Collection as *A. oryzae* (ATTC no. 20386).

Reduction of bitter taste of protein hydrolysates

EP 65,663 and EP 325,986 (Miles Inc.) concerns enzymatic hydrolysis of proteins using a mixture of enzymes containing *Aspergillus oryzae* derived proteases. The obtained protein hydrolysate has a bland, non-bitter taste.

JP-4-7029577 (Asahi Electro-chemical Co.) concerns a protease, derived from *Aspergillus oryzae*, which does not produce any bitter component when decomposing protein.

Prior art discloses a plethora of enzymes exhibiting peptidase, aminopeptidase and other enzyme activities. Said enzymes may be derived from a number of microorganisms, including the fungus species *Aspergillus oryzae*.

In general products, useful for producing protein hydrolysates without a bitter taste, comprise a mixture of peptidase and aminopeptidase activities.

It would therefore be desirable to be able to provide a single-component enzyme (*i.e.* substantially without any side activity) exhibiting only an activity useful for reducing the bitterness of protein hydrolysates used in food products.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the result of SDS-PAGE analysis of supernatant from the *Aspergillus oryzae* A01568 35 kDa aminopeptidase producing transformant.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a single-component enzyme exhibiting an activity, which is particularly useful for preparing improving bread products and for producing proteins and/or protein hydrolysates without bitter taste for foodstuff.

The present inventors have surprisingly succeeded in isolating a DNA sequence encoding an enzyme exhibiting aminopeptidase activity, which advantageously may be used for improving the flavour, crust colour, crumb structure and dough stickiness of baked products. Further, said novel enzyme is useful for producing protein or protein hydrolysates without bitter taste.

The complete DNA sequence encoding said aminopeptidase makes it possible to prepare single-component aminopeptidases.

The complete DNA sequence, shown in SEQ ID no. 1, encoding the aminopeptidase of the invention has, comprised in a

plasmid, been transformed into the bacteria strain *Escherichia coli* DSM no. 9965. This will be described further below.

By a database alignment search it was found that the DNA sequence shown in SEQ ID No. 1 is novel. The highest degree of similarity and identity was found to be 53 % and 32 %, respectively, to the above mentioned 30 kDa aminopeptidase from the bacteria *Vibrio proteolyticus* (ATCC No. 15338).

The inventors have characterized the precursor-form of the aminopeptidase consisting of a secretion signal and the 35 kDa aminopeptidase. The molecular weight (M_w) of the precursor-form was calculated to 41 kDa, and the isoelectric point (pI) was estimated to be approximately 4.9. Further, the amino acid composition of the aminopeptidase was estimated as shown in Table 1.

Table 1

Non-polar:		No.	Percent
	Ala	37	9.79
	Val	23	6.08
	Leu	29	7.67
	Ile	20	5.29
	Pro	14	3.70
	Met	3	0.79
	Phe	19	5.03
	Trp	2	0.53
Polar:		**No.**	**Percent**
	Gly	28	7.41
	Ser	32	8.47
	Thr	25	6.61
	Cys	4	1.06
	Tyr	13	3.44
	Asn	11	2.91
	Gln	16	4.23
Acidic:		**No.**	**Percent**
	Asp	29	7.67
	Glu	25	6.61
Basic:		**No.**	**Percent**
	Lys	28	7.41
	Arg	9	2.38
	His	10	2.65

The deduced complete precursor-form of the amino acid sequence of the 35 kDa enzyme is shown in SEQ ID No. 2.

Accordingly, the first aspect of the invention relates to an enzyme exhibiting aminopeptidase activity having an apparent molecular weight (M_w) of about 35 kDa determined by SDS-PAGE.

Mass spectrometry showed that the average mass of the recombinant aminopeptidase is in the range from 33 kDa to 35 kDa. The isoelectric point (pI) of the enzyme was determined to be about 4.9.

The isoelectric point, pI, is defined as the pH value where the enzyme molecule complex (with optionally attached metal or other ions) is neutral, *i.e.* the sum of electrostatic charges (net electrostatic charge, NEC) on the complex is equal to zero. In this sum of course consideration of the positive or negative nature of the electrostatic charge must be taken into account.

In the following the terms "35 kDa aminopeptidase" and "the enzyme exhibiting aminopeptidase activity" are used interchangeably for the single-component enzyme of the present invention.

The enzyme exhibiting aminopeptidase activity of the invention may be derived from a number of microorganisms. The present inventors have isolated the aminopeptidase of the invention from the filamentous fungus *Aspergillus oryzae* A01568, which is a strain deposited at the American Type Culture Collection as *Aspergillus oryzae* 460 (FERM-P no. 1149, ATCC no. 20386, and further described in US patent no. 3,914,436.

The enzyme exhibiting aminopeptidase activity of the invention comprises at least one of the partial amino acid sequences shown in SEQ ID Nos. 6, 7, 8, 9, and 10, respectively. SEQ ID No 11 is a peptide (5) which overlaps and extends the N-terminal sequence.

In the second aspect, the invention relates to a DNA construct comprising a DNA sequence encoding said aminopeptidase, which DNA sequence comprises

a) the aminopeptidase encoding part of the DNA sequence shown in SEQ ID No. 1, and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

b) an analogue of the DNA sequence shown defined in a), which

i) is homologous with the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

ii) hybridizes with the same oligonucleotide probe as the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified aminopeptidase encoded by the DNA sequence shown in SEQ ID No 1 derived from *Aspergillus oryzae* A01568 or obtainable from *E. coli*, DSM 9965.

In the present context, the "analogue" of the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965, is intended to indicate any DNA sequence encoding an enzyme exhibiting aminopeptidase activity, which has at least one of the properties i)-iv).

The analogous DNA sequence

- may be isolated from another or related (*e.g.* the same) organism producing the enzyme exhibiting aminopeptidase activity on the basis of any of the DNA sequences shown in SEQ ID Nos. 3-5, *e.g.* using the procedures described herein, and thus, *e.g.* be an allelic or species variant of the DNA sequence comprising the DNA sequences shown herein,

- may be constructed on the basis of any of the DNA sequences shown in SEQ ID Nos. 3-5, *e.g.* by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the aminopeptidase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. However, in the latter case amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the polypeptide, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al., (1991), Protein Expression and Purification **2**, 95-107. Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the

function of the molecule and still result in an active enzyme. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, (1989), Science **244**, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resulting mutant molecules are tested for biological (*i.e.* aminopeptidolytic) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labelling. See, for example, de Vos et al. (1992), Science **255**, 306-312; Smith et al., (1992), J. Mol. Biol., **224**, 899-904; Wlodaver et al., (1992), FEBS Lett., **309**, 59-64.

It will be understood that the DNA sequences shown in SEQ ID Nos. 3-5 are sequences which may be used for isolating the entire DNA sequence encoding the aminopeptidase, *e.g.* the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence transformed into the deposited strain *E. coli* DSM 9965. The term "analogue" is intended to include said entire DNA sequence, which comprises one or more of the partial sequences shown in SEQ ID Nos. 3-5 or parts thereof. The amino acid sequence (as deduced from the DNA sequence shown in SEQ ID No. 1) is shown in SEQ ID No. 2.

The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, p. 443-453). Using GAP with

the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least at least 70%, preferably at least 80%, especially at least 90%, with the coding region of the DNA sequence shown in SEQ ID No. 1 or the DNA sequence obtainable from the plasmid in *E. coli* DSM 9965.

The hybridization referred to in ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the aminopeptidase under certain specified conditions which are described in detail in the Materials and Methods section hereinafter.

Normally, the analogous DNA sequence is highly homologous to the DNA sequence such as at least 60% homologous to the DNA sequence shown in SEQ ID No. 1 or the DNA sequence obtainable from the plasmid in *E. coli* DSM 9965 encoding an aminopeptidase of the invention, such as at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or even at least 95% homologous to said DNA sequence.

The homology referred to in iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, p. 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least at least 70%, preferably at least 80%, especially at least 90%, with the coding region of the DNA sequence shown in SEQ ID No. 1 or the DNA sequence obtainable from the plasmid in *E. coli* DSM 9965.

The term "derived from" in connection with property iv) above is intended not only to indicate an aminopeptidase produced by strain A01568, but also an aminopeptidase encoded by a DNA sequence isolated from strain A01568 and produced in a host organism transformed with said DNA sequence. The immunological reactivity may be determined by the method described in the "Materials and Methods" section below.

In further aspects the invention relates to an expression vector harbouring a DNA construct of the invention, a cell comprising the DNA construct or expression vector and a method of producing an enzyme exhibiting aminopeptidase activity which method comprises culturing said cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.
It is also an object of the invention to provide an enzyme preparation enriched with the 35 kDa aminopeptidase of the invention.

Further, the invention provides a bread-improving or a dough-improving composition comprising an enzyme exhibiting aminopeptidase activity of the invention. Said composition may be combined with other enzymes, such as amylolytic enzymes, and conventional bread improving agents.

In a still further aspect the invention relates to a method for preparing a baked product and frozen dough comprising the 35 kDa aminopeptidase of the invention.

Finally the invention relates to the use of the 35 kDa aminopeptidase of the invention. The enzyme of the invention or a composition of the invention comprising such an enzyme may be used for improving the flavour, crust colour and crumb structure of baked products and to improve the stickiness of frozen dough. The aminopeptidase of the invention may furthermore be used advantageously in connection with producing proteins and protein hydrolysates without bitter taste and

may be used for a number of purposes including, degradation or modification of protein containing substances; cleaning of contact lenses, preparation of food and animal feed etc.

DETAILED DESCRIPTION OF THE INVENTION

The DNA sequence of the invention encoding an enzyme exhibiting aminopeptidase activity may be isolated by a general method involving

- cloning, in suitable vectors, a DNA library from *Aspergillus oryzae*,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the DNA library,
- screening for positive clones by determining any aminopeptidase activity of the enzyme produced by such clones, and
- isolating the DNA coding an enzyme from such clones.

The general method is further disclosed in WO 93/11249 the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in Example 3 below.

Microbial Sources

The DNA sequence coding for the aminopeptidase of the invention may for instance be isolated by screening a cDNA library of the donor organism, and selecting for clones expressing the appropriate enzyme activity (*i.e.* aminopeptidase activity as defined by the ability of the enzyme to hydrolyse Leucine-7 amido-4-methylcoumarin). The appropriate DNA sequence may then be isolated from the clone by standard procedures, *e.g.* as described in Example 1.

The donor organism may be a fungus of the *Aspergillus oryzae* (ATCC no. 20386) described in US patent no. 3,914,436

The complete full length DNA sequence encoding the aminopeptidase of the invention has been transformed into a strain of the bacteria *E. coli*, comprised in the expression plasmid pYES 2.0 (Invitrogen). Said bacteria has been deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH., Mascheroder Weg 1b, D-38124 Braunschweig Federal Republic of Germany, (DSM).

Deposit date	: 11.05.95
Depositor's ref.	: NN49001
DSM designation	: *E. coli* DSM No. 9965

Being an International Depository Authority under the Budapest Treaty, Deutshe Sammlung von Mikroorganismen und Zellkulturen GmbH., affords permanence of the deposit in accordance with the rules and regulations of said treaty, *vide* in particular Rule 9. Access to the two deposits will be available during the pendency of this patent application to one determined by the Commisioner of the United States Patent and Trademark Office to be entitled thereto under 37 C.F.R. Par. 1.14 and 35 U.S.C. Par. 122. Also, the above mentioned deposits fulfil the requirements of European patent applications relating to micro-organisms according to Rule 28 EPC.

The above mentioned deposit represents a substantially pure culture of the isolated bacteria. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of the deposited strain does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The DNA sequence encoding the enzyme exhibiting aminopeptidase activity can for instance be isolated from the above mentioned deposited strain by standard methods.

It is expected that a DNA sequence coding for a homologous enzyme, *i.e.* an analogous DNA sequence, is obtainable from other microorganisms. For instance, the DNA sequence may be derived by similarly screening a cDNA library of another microorganism, in particular a fungus, such as a strain of an *Aspergillus sp.*, in particular a strain of *A. aculeatus* or *A. niger*, a strain of another *Trichoderma sp.*, in particular a strain of *T. reesei*, *T. viride*, *T. longibrachiatum* or *T. koningii* or a strain of a *Fusarium sp.*, in particular a strain of *F. oxysporum*, or a strain of a *Humicola sp.*

Alternatively, the DNA sequence coding for an enzyme exhibiting aminopeptidase activity of the invention may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such as any of the above mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of any of the nucleotide sequences shown in SEQ ID Nos. 3-5 or the amino acid sequence shown in SEQ ID No. 2 or any suitable subsequence thereof.

The DNA sequence may subsequently be inserted into a recombinant expression vector. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.* a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and

replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the aminopeptidase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the aminopeptidase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

The host cell which is transformed with the DNA sequence encoding the enzyme of the invention is preferably an eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Aspergillus*, most preferably *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, *e.g.* a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp. *Pichia* sp., *Yarrowia* sp. such as *Yarrowia lipolytica*, or *Kluyveromyces* sp. such as *Kluyveromyces lactis*.

A method of producing an enzyme of the invention

In a still further aspect, the present invention relates to a method of producing an enzyme according to the invention, wherein a suitable host cell transformed with a DNA sequence encoding the enzyme is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed aminopeptidase may conveniently be secreted into the culture medium and may be recovered there from by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Enzyme preparation

In a still further aspect, the present invention relates to an enzyme preparation useful for reducing the bitterness of proteins and/or protein hydrolysates for foodstuff.

The enzyme preparation, having been enriched with an enzyme of the invention, may *e.g.* be an enzyme preparation comprising multiple enzymatic activities, such as an enzyme preparation comprising multiple enzymes for producing protein hydrolysates. The preparation to be enriched can be Flavourzyme® (available from Novo Nordisk A/S). Flavourzyme® is a protease/peptidase complex derived from *Aspergillus oryzae* developed for hydrolysis of proteins.

Dependent on the use for which the enzyme preparation is to be used the aminopeptidase of the invention may be combined with other enzyme as mentioned below.

In the present context, the term "enriched" is intended to indicate that the aminopeptidase activity of the enzyme preparation has been increased, *e.g.* with an enrichment factor of at least 1.1, preferable between 1.1 and 10, more preferred between 2 and 8, especially between 4 and 6, conveniently due to addition of an enzyme of the invention prepared by the method described above.

Alternatively, the enzyme preparation enriched with an enzyme exhibiting aminopeptidase activity may be one which comprises an enzyme of the invention as the major enzymatic component, *e.g.* a single-component enzyme preparation.

The enzyme preparation may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry preparation. For instance, the enzyme preparation may be in the form of a granulate or a microgranulate. The enzyme to be included in the preparation may be stabilized in accordance with methods known in the art.

In another aspect the invention relates to a bread-improving or a dough-improving composition comprising an aminopeptidase of the invention. Said composition may further comprise enzymes selected from the group including amylolytic enzyme, such as α-amylase, β-amylase, maltogenic α-amylase, amyloglucosidase, acid stable amylase, and 1,6-pullulanase.

Such enzymes are available from Novo Nordisk A/S as AMG™ (amyloglucosidase) obtained from a strain of *Aspergillus niger*, Fungamyl™ (fungal amylase) obtained from a strain of *Aspergillus oryzae*, Novamyl™ (maltogenic amylase) obtained from a strain of *Bacillus stearothermophilus*.

The composition of the invention may also comprise one or more additional enzymes. Examples of such enzymes include a cellulase, a hemicellulase, a pentosanase (useful for the partial hydrolysis of pentosans which increases the extensi-

bility of the dough), a lipase (useful for modification of lipids present in the dough or dough constituents so as to soften the dough), a peroxidase (useful for improving the dough consistency), an oxidase, *e.g.* a glucose oxidase, a laccase, a xylanase, a protease (useful for gluten weakening, in particular when using hard wheat flour).

The other enzyme components are preferably of microbial origin and may be obtained by conventional techniques used in the art as mentioned above.

The enzyme(s) to be used in the present invention may be in any form suited for the use in question, *e.g.* in the form of a dry powder or granulate, in particular a non-dusting granulate, a liquid, in particular a stabilized liquid, or a protected enzyme. Granulates may be produced, *e.g.* as disclosed in US 4,106,991 and US 4,661,452 (both to Novo Industri A/S), and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding nutritionally acceptable stabilizers such as a sugar, a sugar alcohol or another polyol, lactic acid or another organic acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

Normally, for inclusion in pre-mixes or flour it is advantageous that the enzyme(s) is/are in the form of a dry product, *e.g.* a non-dusting granulate, whereas for inclusion together with a liquid it is advantageously in a liquid form.

In addition or in an alternative to other enzyme components, the dough-improving and/or bread-improving composition may comprise a conventionally used baking agent, *e.g.* one or more of the following constituents:

A milk powder (providing crust colour), gluten (to improve the gas retention power of weak flours), an emulsifier (to improve dough extensibility and to some extent the consist-

ency of the resulting bread), granulated fat (for dough softening and consistency of bread), an oxidant (added to strengthen the gluten structure; *e.g.* ascorbic acid, potassium bromate, potassium iodate or ammonium persulfate), an amino acid (*e.g.* cysteine), a sugar, and salt (*e.g.* sodium chloride, calcium acetate, sodium sulfate or calcium sulphate serving to make the dough firmer), flour or starch. Such components may also be added directly to the dough in accordance with a method of the invention.

Examples of suitable emulsifiers are mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, phospholipids and lecithin.

The bread-improving and/or dough improving composition of the invention is typically included in the dough in an amount corresponding to 0.01-5%, in particular 0.1-3%.

In accordance with the method of the invention, in which an enzyme with aminopeptidase activity of the invention, optionally in combination with other enzymes as described above, is used for the preparation of dough and/or baked products, the enzyme(s) may be added as such to the mixture from which the dough is made or to any ingredient, *e.g.* flour, from which the dough is to be made. Alternatively, the enzyme(s) may be added as a constituent of a dough-improving and/or a bread-improving composition as described above, either to flour or other dough ingredients or directly to the mixture from which the dough is to be made.

The dosage of the enzyme(s) to be used in the method of the present invention should be adapted to the nature and composition of the dough in question as well as to the nature of the enzyme(s) to be used. Normally, the enzyme preparation is

added in an amount corresponding to 0.01-1000 mg enzyme protein per kg of flour, preferably 0.1-100 mg enzyme protein per kg of flour, more preferably 0.1-10 mg enzyme protein per kg of flour.

In terms of enzyme activity, the appropriate dosage of a given single component enzyme with aminopeptidase activity, optionally in combination with other enzyme(s), for exerting a desirable improvement of flour or crust colour of a baked product will depend on the enzyme(s) and the enzyme substrate(s) in question. The optimal dosage may vary dependent on the flour or yeast types and baking process. The skilled person may determine a suitable enzyme unity dosage on the basis of methods known in the art.

However, according to the present invention the enzyme exhibiting aminopeptidase activity of the invention is added in an amount corresponding 30 to 1000 LAPU, preferably 50 to 500 LAPU, especially 80 to 300 LAPU, such as about 100 LAPU per kg of flour. LAPU is defined below.

Amylolytic enzymes are normally added in from 1 to 50 FAU per kg flour. One FAU (Fungal α-Amylase Unit) is the amount of enzyme which breaks down 5.26 g starch (Merck, Amylum solubile Erg. B.6, BAch 9947275) per hour using Novo Nordisk's standard method for determination of αamylase activity. A detailed description of Novo Nordisk's method (AF 216) of analysis is available on request.

Maltogenic amylase are normally added in from 1 to 1000 MANU per kg flour (Maltogenic Amylase Novo Units). One MANU is defined as the amount of enzyme which, under standard conditions, hydrolyzes i micromole of maltotriose per minute. The analytic method (AF 203) is available on request.

When one or more additional enzyme activities are to be added in accordance with the method of the invention, these activities may be added separately or together with the singes

component enzyme with exopeptidase activity, optionally as constituent(s) of the bread-improving and/or dough-improving composition of the invention. The other enzyme activities may be any of the above described enzymes and may be dosed in accordance with established baking practice.

As mentioned above the enzyme exhibiting aminopeptidase activity, optionally in combination with other enzyme(s) as described above, is added to any mixture of dough ingredients, to the dough, or to any of the ingredients to be included in the dough, in other words the enzyme(s) may be added in any step of the dough preparation and may be added in one, two or more steps, where appropriate.

The handling of the dough and/or baking is performed in any suitable manner for the dough and/or baked product in question, typically including the steps of kneading the dough, subjecting the dough to one or more proofing treatments, and baking the product under suitable conditions, *i.e.* at a suitable temperature and for a sufficient period of time. For instance, the dough may be prepared by using a normal straight dough process, a sour dough process, an overnight dough method, a low-temperature and long-time fermentation method, a frozen dough method, the Chorleywood Bread process, or the Sponge and Dough process.

The dough and/or baked product prepared by the method of the invention are normally based on wheat meal or flour, optionally in combination with other types of meal or flour such as corn flour, rye meal, rye flour, oat flour or meal, soy flour, sorghum meal or flour, or potato meal or flour.

In the present context the term "baked product" is intended to include any product prepared from dough, either of a soft or a crisp character. Examples of baked products, whether of a white, light or dark type, which may advantageously be produced by the present invention are bread (in particular

white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pita bread, tacos, cakes, pan-cakes, biscuits, crisp bread and the like.

The dough of the invention may be of any of the types discussed above, and may be fresh or frozen.

The preparation of frozen dough is described by K. Kulp and K. Lorenz in "Frozen and Refrigerated Doughs and Batters". When using the aminopeptidase of the invention for frozen bread the flavour, the crust colour and the crispiness are improved.

From the above disclosure it will be apparent that the dough of the invention is normally a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways such as by adding sodium bicarbonate or the like or by adding a leaven (fermenting dough), but it is preferred to leaven the dough by adding a suitable yeast culture such as a culture of *Saccharomyces cerevisiae* (baker's yeast). Any of the commercially available *S. cerevisiae* strains may be employed.

As mentioned above, the present invention further relates to a pre-mix, *e.g.*, in the form of a flour composition, for dough and or baked products made from dough, which pre-mix comprises an enzyme exhibiting aminopeptidase activity of the invention and optionally other enzymes as specified above. The pre-mix may be prepared by mixing the relevant enzyme(s) or a bread-improving and/or dough-improving composition of the invention comprising the enzyme(s) with a suitable carrier such as flour, starch, a sugar or a salt. The pre-mix may contain other dough-improving and/or bread-improving additives, *e.g.*, any of the additives, including enzymes, mentioned above.

Use of the 35 kDa Aminopeptidase of the invention

Use of the Enzyme of the invention for preparing Baked Products

The enzyme or an enzyme preparation of the invention may be used in baking, *e.g.* in order to weaken the gluten components of flour so as to obtain a softening of so-called hard flour.

However, it has surprisingly been found that the aminopeptidase of the invention does not degrade the network of the gluten which is normally observed when proteases are used for preparing baked products. Consequently, the dough characteristics and crumb structure are not affected.

Further, when adding the 35 kDa aminopeptidase of the invention to the dough or dough ingredients, when preparing baked products, the flavour crust colour and/or the crumb structure and/or the crust colour of a baked product will be substantially improved, as shown in Example 13.

Further, the enzyme of the invention improves the dough stickiness.

The addition of the enzyme of the invention results in baked products having a flavour of yeasty type, providing a "freshly baked" bread smell. This make the invention of particular interest for the sponge-dough-system, in which an addition of the enzyme can reduce the sponge fermentation time without a concomitant loss of yeasty flavour, and in the no-time-dough process (most European processes), in which the enzyme can provide a yeasty flavour which is otherwise normally lacking products prepared from such processes.

Without being limited to any theory it is presently believed that further improved flavour and/or crust colour of a baked product may be obtained when a single component enzyme with exopeptidase activity is used in combination with an amylolytic enzyme, in particular an amylolytic enzyme which is

capable of liberating reducing sugar molecules from flour or other constituents of the dough. The increased amount of reducing sugars in the dough provides an increase in Maillard reactions taking place during baking thereby further improving the flavour and crust colour of the baked product.

Use of the Enzyme of the invention for Reducing Bitter Taste

The enzyme or enzyme preparation of the invention may be used for reducing the bitterness of proteins and/or protein hydrolysate for foodstuff.

Also contemplated according to the invention is the production of free amino acids from proteins and/or protein hydrolysates. In the case of the free amino acid are glutamine acid it enhances the flavour of food products.

Said protein or protein hydrolysate may be of animal or vegetable origin.

In an embodiment of the invention the protein to be hydrolysed is casein or soy protein.

The protein may be use for producing foodstuff such as cheese and foodstuff containing cocoa.

Even though the aminopeptidase and enzyme preparations enriched with an enzyme of the invention may be used especially advantageously in connection with producing proteins or protein hydrolysates without bitter taste, the aminopeptidase of the invention can be used for a number of industrial applications, including degradation or modification of protein containing substances, such cell walls. Some proteins, like extensins, are components of plant cell walls. Aminopeptidases will therefore facilitate the degradation or modification of plant cell walls.

The dosage of the enzyme preparation of the invention and other conditions under which the preparation is used may be determined on the basis of methods known in the art.

Extraction of Oil from Plants

The enzyme preparation according to the invention may be useful for extraction of oil from plant sources like olives and rape or for production of juice from different fruits like apples, pears and citrus. It may also be useful in the wine industry, especially in the white wine industry, to prevent haze formation. Furthermore, it may be used to modify and degrade proteins, *e.g.* in order to reduce the viscosity caused or partially caused by proteins, or to facilitate fermentative processes where proteins are involved, or it may be used to improve the digestibility of proteins and other nutrients.

The Use for preparing Food and Feed

The aminopeptidase preparation may also be used in the food and feed industry to improve the digestibility of proteins. For instance, the enzyme or enzyme preparation may be added to animal feed or may be used to process animal feed, in particular feed for piglets or poultry.

Further the enzyme or enzyme preparation of the invention may be useful to make protein hydrolysates from, *e.g.*, vegetable proteins like soy, pea, lupin or rape seed protein, milk like casein, meat proteins, or fish proteins. The aminopeptidase may be used for protein hydrolysates to improve the solubility, consistency or fermentability, to reduce antigenicity or for other purposes to make food, feed or medical products. The aminopeptidase may be used alone or together with other aminopeptidases or together with other enzymes like exopeptidases. The use of the aminopeptidase of the invention together with exopeptidase rich enzyme preparations will improve the taste of the protein hydrolysates.

Furthermore, the enzyme or enzyme preparation may be used in the processing of fish or meat, *e.g.* to change texture and/or viscosity.

Use in Brewing processes

The enzyme preparation may also be used to facilitate fermentative processes, like yeast fermentation of barley, malt and other raw materials for the production of *e.g.* beer.

Use for making Protoplast

The enzyme preparation may be useful for making protoplasts from fungi.

Use for the production of Peptides

The enzyme preparation may be useful for production of peptides from proteins, where it is advantageous to use a cloned enzyme essentially free from other proteolytic activities.

Use for degradation of Proteins

Further, the aminopeptidase preparation can be used to degrade protein in order to facilitate purification of or to upgrade different products, like in purification or upgrading of gums, like guar gum, xanthan gum, degumming of silk, or improvement of the quality of wool.

Use for cleaning Contact Lenses

Further the enzyme or enzyme preparation may be used for cleaning of contact lenses.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

METHODS AND MATERIALS

Materials

Donor organism: *Aspergillus oryzae* A01568 (described in US patent no. 3,914,436)

Host organism:

Escherichia coli MC1061 (Meissner et al., (1987), Proc. Natl. Acad. Sci. U.S.A., **84**, 4171-4176: cDNA library strain

Saccharomyces cerevisiae W3124 (van den Hazel et al., (1992), Eur. J. Biochem., **207**, 277-283): Activity screening strain.

Schizosaccharomyces pombe: Bröker et al., (1989), FEBS Letters, **248**, 105-110.

Other organisms:

Aspergillus oryzae A1560 (Christensen et al. (1988), Bio/technology **6**, 1419-1422).

Plasmids:

pYES 2.0: Transformation vector (Invitrogen).

pHD414: *Aspergillus* expression vector is a derivative of the plasmid p775 (described in EP 238.023). The construction of the pHD414 is further described in WO 93/11249. pHD414 contains the *A. niger* glucoamylase terminator and the *A. oryzae* TAKA amylase promoter.

pHD423 is a derivative of pHD414 (described in WO 94/20611) with a new polylinker.

pUC18: Expression vector (Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual, 2. ed.; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York)

pC1EXP3: plasmid comprising the 1.4 kb cDNA insert encoding the 35 kDa aminopeptidase of the invention.(See Example 3 and SEQ ID NO 1)

pC1EXP4: plasmid comprising a cDNA sequence, which is 120 bp shorter that the pC1EXP3 1.4 kb cDNA insert. (see Example 10 and SEQ ID NO 12)

p3SR2: *A. nidulans* amdS+ gene carrying plasmid (Christensen et al., (1988), Bio/Technology **6**, 1419-1422)

pP1: yeast expression vector, which is an *E. coli/S. pombe* shuttle vector containing the ADH promoter and URA 3 as selective marker (Bröker et al., (1989), FEBS Letters, **248**, 105-110).

Primers:

Universal pUC primers (Sambrook et al., (1989), supra)

Deduced primer sequences used in PCR reactions

s2:

5'- GAR ACI GTI CAR AAY CTI AT -3'

s3:

5'- GAY AAR AAR AAY TTY GAW ACI GT -3'

as1:

5' - TCI ACR TTR TCI GTI ATI ATY TCI AT -3'

(s=sense, as= anti-sense)

A = Adenine

G = Guanine

C = Cytosine

T = Thymine

I = Deoxyinosine

Y = C or T
R = A or G
W = A or T

Forward and Reverse pYES primers (Invitrogen)

Enzymes:
Lysine-specific protease
Novozym® 234 (Novo Nordisk A/S)
Alcalase® (Novo Nordisk A/S)
Neutrase® (Novo Nordisk A/S)

Peptides of the 35 kDa aminopeptidase:
(see SEQ ID No. 6-11)

N-terminal:

Direct N-terminal sequencing of authentic and recombinant aminopeptidase revealed the same N-terminal amino acid sequence for the two enzymes showing that the recombinant enzyme is proteolytically processed identical to the authentic enzyme. The N-terminal sequence found was

Tyr-Pro-Asp-Ser-Val-Gln-His-Xaa-Glu-Thr-Val-Gln-Asn-Leu-Ile-
s2 ------>
Lys-Ser-Leu-Asp-Lys-Lys-Asn-Phe-Glu-Thr-Val-Leu-Gln-Pro-
s3------>

(Xaa is a glycosylated Asn-residue)

The following peptide sequence was obtained from peptides derived from a S-carboxymethylated sample of the aminopeptidase by cleavage with a lysyl-specific protease.

Peptide 1:

Tyr-Pro-Asp-Ser-Val-Gln-His-Xaa-Glu-Thr-Val-Gln-Asn-Leu-Ile-Lys

Peptide 2:

Gly-Val-Thr-Val-Glu-Pro-Phe-Lys

Peptide 3:

Val-Ile-Val-Asp-Ala-Tyr-Cys-Thr-Ile-Pro-Thr-Val-Asp-Ser-Lys

Peptide 4:

Gly-Thr-Thr-Asp-Ala-Gly-Lys-Pro-Glu-Ser-Ile- Glu-Ile-Ile-Thr-
<-------asl
Asp-Asn-Val-Asp-Glu-Asn-Leu-Thr-Lys

Peptide 5
Asn-Phe-Glu-Thr-Val-Leu-Gln-Pro-Phe-Ser-Glu-Phe-His-Asn-Arg-
Tyr-Tyr-Lys

(Overlaps and extends the N-terminal sequence).

Media and other materials:

STC: 1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5., 10 mM $CaCl_2$

BSA (Sigma, type H25)

Leucine-7 amido-4-methylcoumarin (Sigma)

PEG 4000 (polyethylene glycol, molecular weight = 4,000) (BDH, England)

Sequenase®Kit (United Stated Biochemical, USA)

Hybond-N nylon membrane (Amersham, USA)

Q-sepharose (Pharmacia Tm)

Superdex 200 Tm

Amicon membrane

VG Analytical TofSpec

α-Cyano-4-hydroxycinnamic acid (Aldrich, Steinheim, Germany).

YPD: 10 g yeast extract, 20 g peptone, H_2O to 810 ml. Autoclaved, 90 ml 20% glucose (sterile filtered) added.

10 x Basal salt: 66.8 g yeast nitrogen base, 100 g succinic acid, 60 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20% casamino acids, 9 ml 1% tryptophan, H_2O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose or 20% galactose added.

SC-H broth: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan. Autoclaved for 20 min. at 121°C. After autoclaving, 10 ml of a 30% galactose solution, 5 ml of a 30% glucose solution and 0.4 ml of a 5% threonine solution were added per 100 ml medium.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan, and 20 g/l agar (Bacto). Autoclaved for 20 min. at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

YNB-1 agar: 3.3 g/l KH_2PO_4, 16.7 g/l agar, pH adjusted to 7. Autoclaved for 20 min. at 121°C. After autoclaving, 25 ml of a 13.6% yeast nitrogen base without amino acids, 25 ml of a 40% glucose solution, 1.5 ml of a 1% L-leucine solution and 1.5 ml of a 1% histidine solution were added per 450 ml agar.

YNB-1 broth: Composition as YNB-1 agar, but without the agar.

Minimal plates: (Cove Biochem.Biophys.Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl

Whey protein hydrolysate: Whey hydrolysed with Alcalase® and Neutrase® was diluted with water until the protein content was 8% (w/w) of the total solution.

Methods

RNA isolation: The total RNA was prepared, from frozen, powdered mycelium of *A. oryzae* A01568, by extraction with

guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin et al., (1979), Biochemistry **18**, 5294-5299). The poly(A)$^+$RNA was performed by oligo(dT)-cellulose affinity chromatography (Aviv, H, and Leder, P., (1972), Proc. Natl. Acad. Sci. U.S.A. **69**, 1408-1412).

cDNA synthesis: Double-stranded cDNA was synthesized from 5 µg of *Aspergillus oryzae* poly(A)$^+$ RNA as described by Kofod et al., J. of Biol. Chem., (1994), **269**, 29182-29189, except that 25 ng of random hexanucleotide primers (Pharmacia, Sweden) were included in the first strand synthesis.

Construction of cDNA library:
The cDNA library was constructed as described by Kofod et al., J. of Biol. Chem., (1994), **269**, 29182-29189.

Transformation of *Saccharomyces cerevisiae*:
To ensure that all the bacterial clones were tested in yeast, a number of yeast transformants 5 times larger than the number of bacterial clones in the original pools was set as the limit.

One µl aliquots of purified plasmid DNA (100 ng/µl) from individual pools were electroporated (200 Ω, 1.5 kV, 24 µF) into 40 µl of competent *S. cerevisiae* cells (OD600 = 1.5 in 500 ml YPD, washed twice in cold distilled water, once in cold 1 M sorbitol, resuspended in 0.5 ml 1 M sorbitol, Becker & Guarante, 1991). After addition of 1 ml 1 M cold sorbitol, 80 µl aliquots were plated on SC + glucose - uracil to give 250-400 c.f.u./plate and incubated at 30°C for 3-5 days.

Transformation of *Schizosaccharomyces pombe*
Schizosaccharomyces pombe was transformed as described by Bröker, (1987) BioTechniques, **5**, 51-517.

Purification of 35 kDa aminopeptidase from *Aspergillus oryzae*:

One gram freeze dried powder of the fermentation supernatant of *Aspergillus oryzae* A01568 was dissolved in 100 ml Tris-acetate buffer (25 mM pH 8). Ionic strength was 2 mSi. The suspension was filtered through 45 μ millipore filter. The filtered solution was applied on a 200 ml anion exchange chromatography column packed with Q-sepharose, which was equilibrated with the Tris-acetate buffer. Alkaline protease which is a major endoprotease with isoelectric point of 8 was collected in effluent. The column was washed with the Tris-acetate buffer until no more UV absorbing material was present in effluent.

The bound proteins were eluted with linear salt gradient using 0 to 0.5 M NaCl in the Tris-acetate buffer (pH 8) using 10 column volume with a flow rate of 4 ml/minutes. Fractions containing aminopeptidase activity (see below) were pooled and dialyzed against Tris-acetate buffer (25 mM , pH 6).

The dialyzed pool containing activity was adjusted to pH 6 and ionic strength to 2 mSi and applied on 50 ml High performance Q-sepharose column, equilibrated with 25 mM Tris-acetate buffer pH 6, for anion exchange chromatography. The column was then washed until the UV absorbing material in effluent was under 0.05 at 280 nm. Bound activity was then eluted with 20 column volume linear salt gradient, from 0 to 0.5 M NaCl at a flow rate of 2 ml/minutes. Fractions containing aminopeptidase activity were pooled and concentrated by ultrafiltration, using 50 mM sodium-acetate buffer (pH 6).

Two ml of the concentrated pool containing aminopeptidase activity was applied on Superdex 200 Tm column equilibrated with 50 mM sodium acetate buffer (pH 6) containing 0.1 M NaCl. The gel filtration was carried out using a flow rate of 0.5 ml/minutes. Samples containing aminopeptidase activity

were pooled and concentrated by ultrafiltration using Amicon membrane with a cut-off-value of 10 kDa.

Amino acid sequence determination of N-terminal and internal peptides of the *Aspergillus oryzae* aminopeptidase:
S-carboxymethylated samples of the purified native aminopeptidase is digested with lysyl-specific protease, and the resulting peptides are separated by reverse phase high pressure liquid chromatography (HPLC) and sequenced as described by Matsudaira, A Practical Guide to Protein and peptide Purification for Microsequencing, 3-88, Academic Press Inc, San Diego, CA, in an Applied Biosystems 473A sequencer according to the manufacturer's instructions (Applied Biosystems).

Reagents and solvents for amino acid sequencing are from Applied Biosystems (Foster City, CA).

Designing of PCR primers:
The PCR primers were designed as described by Kofod et al., J. of Biol. Chem., (1994), **269**, 29182-29189.

Generation of a cDNA probe for an aminopeptidase using PCR:
One µg of double stranded plasmid DNA from a cDNA library pool was PCR amplified using 500 pmol of each of the designed primers in combinations with 500 pmol of pYES 2.0 polylinker primer (forward and reverse), a DNA thermal cycler (Landgraf, Germany) and 2.5 units of *Taq* polymerase (Perkin-Elmer).

Thirty cycles of PCR were performed using a cycle profile of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 3 minutes.

Dideoxy chain-termination method:
The method was carried out as described by Sanger et al., (1977), Proc. Natl. Acad. Sci. U.S.A. **74**, 5463-5467, using the Sequenase®Kit and universal pUC primers.

Characterization of positive cDNA clones by Southern blot analysis:
The positive clones were characterized by the use of Southern blot hybridization using the 0.5 kb random-primed ^{32}P-labelled PCR-product or 35 kDa aminopeptidase as probe. The hybridizations were carried out in 2 x SSC, 5 x Denhardt's solution, 0.5% (w/v) SDS, 100 µg/ml denatured salmon sperm DNA for 48 hours at 65°C followed by washing at high stringency in 2 x SSC (2 x 15 minutes), 2 x SSC, 0.5% SDS (30 minutes), 0.2 x SSC, 0.5% SDS (30 minutes) and finally in 2 x SSC (15 minutes), at 65°C (Sambrook et al. (1989), supra).

Electrophoresis
Electrophoresis was performed on a 0.7% agarose gel from SeaKem, FMC.

Capillary blotting
Capillary blotting method is described by Sambrook et al., (1989), supra) using 10 x SSC as transfer buffer

High stringency washes of hybridized clones:
Washing was carried out in 2 x 15 minutes in 2 x SSC, 2 x 30 minutes in 0.1 x SSC, 0.5% SDS and 15 minutes in 2 x SSC, at 65°C.

Transformation of *Aspergillus oryzae*:
Transformation of *Aspergillus oryzae* was carried out as described by Christensen et al., (1988), Biotechnology **6**, 1419-1422.

Construction of the aminopeptidase expression cassette for *Aspergillus*

Plasmid DNA was isolated from the positive *E. coli* clones using standard procedures and analyzed by restriction enzyme analysis. The cDNA insert was excised using appropriate restriction enzymes and ligated into an *Aspergillus* expression vector.

Transformation of *Aspergillus oryzae* or *Aspergillus niger* (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) is inoculated with spores of *A. oryzae* or *A. niger* and incubated with shaking at 37°C for about 2 days. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$. 10 mM NaH_2PO_4, pH = 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234 is added. After 5 minutes 1 ml of 12 mg/ml BSA is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayered with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation is performed for 15 minutes at 100 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC are added to the protoplast suspension and the mixture is centrifugated for 5 minutes at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated.

Finally the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC. Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid). The

mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000. 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on the appropriate plates. Protoplasts are spread on minimal plates to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the second re-isolation is stored as a defined transformant.

Purification of the *Aspergillus oryzae* transformants:
Aspergillus oryzae colonies are purified through conidial spores on $AmdS^+$-plates (+ 0,01% Triton X-100) and growth in YPM for 3 days at 30° C.

Identification of aminopeptidase positive *Aspergillus oryzae* transformants:
The supernatants from the *Aspergillus oryzae* transformants were assayed for aminopeptidase on agar plates overlayered with 60 µg/ml of Leucine-7 amido-4-methylcoumarin. Positive transformants were identified by analyzing the plates by fluorescence under UV-light after 5 minutes to 2 hours incubation at 30°C.

SDS-PAGE analysis:
SDS-PAGE analysis of supernatant from an *Aspergillus oryzae* aminopeptidase producing transformant. The transformant was grown in 5 ml YPM for three days. 10 µl of supernatant was applied to 12% SDS-polyacrylamide gel which was subsequently stained with Coomassie Brilliant Blue.

Mass spectrometry
Mass spectrometry is done using matrix assisted laser desorption ionisation time-of flight mass spectrometry in a

VG Analytical TofSpec. For mass spectrometry 2 µl of sample is mixed with 2 µl saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile (70:30)) and 2 µl of the mixture spotted onto the target plate. Before introduction into the mass spectrometer the solvent was removed by evaporation. Samples are desorbed and ionised by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions were detected by a micro channel plate set at 1850 V. The spectra is calibrated externally with proteins of known mass.

Immunological cross-reactivity:

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified aminopeptidase. More specifically, antiserum against the aminopeptidase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, *e.g.* on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Southern blot analysis:

Genomic DNA from *A. oryzae* is isolated according to Yelton et al., (1984), Proc. Natl. Acad. Sci. U.S.A. 81., p. 1470-1474,

and digested to completion with BamHI, BglII, EcoRI and HindIII (10 μg/sample), fractionated on a 0.7 % agarose gel, denatured and blotted to a nylon filter (Hybond-N) using 10 x SSC as transfer buffer (Southern, E. M., (1975), J. Mol. Biol. 98, p. 503-517). The aminopeptidase cDNA is ^{32}P-labeled (> 1 x 10^9 cpm/μg) by random-priming and used as a probe in Southern analysis. The hybridization and washing conditions are as described in RNA gel blot analysis. The filter is autoradiographed at -80°C for 12 hours.

RNA gel blot analysis:

Poly(A)$^+$ RNA (1 μg) from *A. oryzae* is electrophoresed in 1.2 % agarose-2.2 M formaldehyde gels (Thomas, P. S., (1983) Methods Enzymol. 100, pp. 255-2663) and blotted to a nylon membrane (Hybond-N) with 10 x SSC as transfer buffer. The aminopeptidase cDNA is ^{32}P-labeled (> 1 x 10^9 cpm/μg) by random priming and hybridized to the membrane for 18-20 hours at 65°C in 5 x SSC, 5 x Denhardt's solution, 0.5% SDS (w/v) and 100 μg/ml denatured salmon sperm DNA. The filter is washed in 5 x SSC at 65°C (2 x 15 minutes), 2 x SSC, 0.5 % SDS (1 x 30 minutes), 0.2 x SSC, 0.5 % SDS (1 x 30 minutes), and 5 x SSC (2 x 15 minutes). The filter is autoradiographed at - 80°C for 12 hours.

Determination of aminopeptidase activity (LAPU)

One LAPU is defined as the amount of enzyme which hydrolyzes 1 μmole of L-leucine-p-nitroanilide per minute using the method described in AF 298/1-GB (available on request from Novo Nordisk A/S).

% DH determination based on TMBS analysis

The extent of protein hydrolysis may be determined by the degree of hydrolysis achieved. In the context of this invention, the degree of hydrolysis (DH) is defined by the following formula:

$$DH = \frac{h}{h_{tot}} \times 100\ \%$$

h is the number of peptide bonds hydrolysed and h_{tot} is the total number of peptide bonds in the protein. h_{tot} is dependent on the type of raw material, whereas h can be expressed as a function of meqv leucine NH_2, measured by for instance TNBS-analyses

Determination of %DH is described in EF-9415317 (available on request from Novo Nordisk A/S)

Testing of Doughs and Breads

According to the present invention the effect of adding a single component enzyme with aminopeptidase activity may be tested in doughs and breads by using the following method:

Preparation of Breads

Procedure:

1. Dough mixing (Spiral mixer)
   3 min. at 700 RPM
   5 min. at 1400 RPM

the mixing time is predetermined and adjusted by a skilled baker based on the flour used so as to obtain an optimum dough consistence under the testing conditions used.

2. 1st proof: 30°C - 80% RH, 15 min.
3. Scaling and shaping;
4. Resting for 5 minutes at ambiant temperature;
5. Final proof: 32°C - 80% RH, 45 minutes for rolls, 55 minutes for bread;
6. Baking: 225°C, 22 minutes for rolls and 30 minutes for loaf.

Evaluation of Dough and Baked Products

Dough and baked products may be evaluated as follows:

Loaf specific volume: the mean value of 4 loaves volume are measured using the traditional rape seed method. The specific

volume is calculated as volume ml per g bread. The specific volume of the control (without enzyme) is defined as 100. The relative specific volume index is calculated as:

$$\text{Specific vol. index} = \frac{\text{specific vol. of 4 loaves}}{\text{spec. vol. of 4 control loaves}} * 100$$

The dough stickiness and crumb structure may be evaluated visually according to the following scale:

Dough stickiness:	almost liquid	1
	too sticky	2
	sticky	3
	acceptable	3.5
	normal	4
	dry	5
Crumb structure:	very poor	1
	poor	2
	non-uniform	3
	uniform/good	4
	very good	5

Shock test: After the second proof a pan containing the dough is dropped from a height of 20 cm. The dough is baked and the volume of the resulting bread is determined.

Yeasty Flavour

as control	3
slightly improved	3.5
Improved	4

Crumb colour

The crumb colour is determined visually

EXAMPLES

Example 1

Construction of cDNA library

Total RNA was extracted from *Aspergillus oryzae* A01568. Poly(A)+RNA was isolated by oligo(dT)-cellulose affinity chromatography and double stranded cDNA (ds cDNA) was synthesized.

A cDNA library from *Aspergillus oryzae* A01568 consisting of 3.5×10^6 clones was constructed into the yeast expression vector pYES 2.0.

Example 2

Amplification and characterization of cDNA clones.

The aminopeptidase was purified from *Aspergillus oryzae* A01568 as described above in the section "Materials and Methods".

A long NH_2-terminal sequence and four internal sequences (including Peptide 1 to 5) of the aminopeptidase were obtained by digestion of S-carboxymethylated purified protein with a lysyl-specific protease.

Based on these sequences three primers were synthesized (as1, s2 and s3, respectively). Double stranded cDNA (ds cDNA) was used as template in the PCR amplification experiment as described above in the section "Materials and Methods".

Analysis of the resulting PCR-products revealed a 0.5 kb fragment with one primer pair (primer s3 and primer as1).

The PCR-fragment was sub-cloned into SmaI-cut dephosphorylated pUC18 vector and sequenced from both ends using the Dideoxy chain-termination method as described above in the section "Material and Methods".

In addition to the primer encoded residues, the sequence of Peptide 2 obtained from the purified aminopeptidase, aligned with the deduced amino acid sequence, confirmed that the desired cDNA species had been specifically PCR amplified.

Example 3

Screening of the cDNA library for clones encoding the aminopeptidase from *Aspergillus oryzae*

Approximately 10,000 colonies from the cDNA library from *Aspergillus oryzae* A01568 were screened by colony hybridization as described above. This yielded positive clones with inserts ranging from 650 bp to 1.5 kb.

The positive clones were analyzed by Southern blot analysis as described above in the section "Materials and Methods".

Purified plasmid DNA (about 1 μg) from the aminopeptidase cDNA clones was digested to completion by HindIII and XbaI to release the cDNA inserts from the pYES vector. The samples were electrophoresed and transferred to a Hybond-N nylon membrane by the capillary blotting method. The filters were washed at high stringency resulting in positive clones with inserts ranging from 900 bp to 1700 bp.

Strongly hybridizing clones were analyzed by sequencing the ends of the cDNAs with forward and reverse pYES primers.

Analysis of the sequence data showed that some of these clones were truncated cDNAs whereas others appeared to be full-length clones. The nucleotide sequence and deduced amino acid sequence of one of the full-length clones (pC1EXP3) obtained (shown in figure 1) contains a cDNA insert of 1.4 kb.

Example 4

Expression of the aminopeptidase in *Aspergillus oryzae*

To obtain high level production of aminopeptidase in *Aspergillus oryzae*, the cDNA insert from the pC1EXP3 clone was sub-cloned into pHD423 and co-transformed with the AmdS$^+$ plasmid into *Aspergillus oryzae* as described above.

The 1.4 kb cDNA insert from pC1EXP3 was isolated from pYES 2.0 by Hind III and Not I digestion, ligated to a Not I/Hind III cleaved pHD 423 vector, and transformed into *E. coli*.

The resulting transformants were purified twice (see above) and assayed for aminopeptidase activity as described above. Transformant pA3EXP3/1 showed detectable aminopeptidase activity.

Example 5

Expression level of aminopeptidase producing transformant (pA3EXP3/1)

The amount and purity (level of expression) of secreted aminopeptidase from the transformant (pA3EXP3/1) was determined semi-quantitatively by SDS-PAGE using the non-transformed *A. oryzae* strain A01560 as a negative control and the *A. oryzae* A01568 strain as positive control (see figure 1).

A 36-37 kDa polypeptide could be seen in pA3EXP3/1, not present in the negative control. The size of the recombinant aminopeptidase is approximately 2 kDa higher than that of the native aminopeptidase (a double band of about 35 kDa), possibly due to additional glycosylation or other types of post-translational modifications.

Example 6

Mass spectrometry showed that the recombinant aminopeptidase is glycosylated as the mass determined exceed the mass of the polypeptide calculated from the cDNA sequence to be 32.4 kDa.

The average mass of the recombinant aminopeptidase is 34.1 kDa with the masses ranging from 33 kDa to 35 kDa.

The apparent molecular weight (M_w) determined by SDS-PAGE (as described in "Materials and Methods" was found to be about 35 kDa.

Example 7

Fermentation of 35 kDa aminopeptidase producing transformant

The *Aspergillus oryzae* transformant pA3EXP3/1 was grown in one liter shake flasks containing 150 ml DAP 2C (pH = 5.9) for three days at 30°C.

The amount of secreted aminopeptidase was estimated by SDS-PAGE analysis to approximately 0.5 g/liter supernatant.

Example 8

Expression of 35 kDa aminopeptidase clones in *S. pombe*

The full-length 35 kDa aminopeptidase cDNA clone pC1EXP3 was re-transformed into *Schizosaccharomyces pombe* by electroporation. The 1.4 kb cDNA insert was released from the pYES 2.0 vector by SpeI and NotI digestion and subcloned into the SpeI/NotI cleaved yeast expression vector pP1, which is an *E. coli/S. pombe* shuttle vector containing the ADH promoter, and assayed for aminopeptidase activity.

It was shown that one *S. pombe* transformant had strong aminopeptidase activity, indicating that *S. pombe* is able to synthesize and secrete a functionally active 35 kDa aminopeptidase from *Aspergillus oryzae* A01568.

Example 9

Organization and Expression of the Aminopeptidase gene

The copy number of the aminopeptidase gene in the *A. oryzae* A01568 genome was determined by Southern blot hybridization described in the "Materials and Methods" section. Total DNA isolated from *A.oryzae* was digested to completion with BamHI, BglII, EcoRI or HindIII and hybridized with the aminopeptida-

se cDNA. The aminopeptidase probe detects only single strongly hybridizing fragments in each case except in BamHI digestion, which gives two hybridizing fragments due to a BamHI site at nucleotide position 640. This indicates that the aminopeptidase gene is present as a single copy in the *A. oryzae* A01568 genome.

Example 10

To study the expression of the 35 kDa aminopeptidase gene, poly $(A)^+$ RNA extracted from *A. oryzae* A01568 mycelium was subjected to Northern blot analysis. Probing of the blotted RNA with the aminopeptidase cDNA revealed two species of mRNA of approximately 1.45 and 1.55 kilobases. These two mRNAs do not appear to represent transcripts of two distinct genes since the same pattern of hybridization was observed when the filters were washed at high stringency. The difference in size of the two mRNAs could be due to different lengths of 3´ untranslated region, because of two polyadenylation sites, in accordance with the two cDNA species isolated from the *Aspergillus oryzae* cDNA library, one corresponding to clones pC1EXP3 and another one corresponding to pC1EXP4, which is 120 bp shorter. Both mRNAs encode the same aminopeptidase.

Example 11

Removing bitter taste from whey protein hydrolysate

The fermentation broth of *Aspergillus oryzae* transformant pA3EXP3/1 was tested for the ability to debitter a solution containing 8% (w/w) protease hydrolysed whey protein.

The aminopeptidase activity of fermentation broth was 5.58 LAPU/g.

The substrate protein solution was tested in flasks containing 100 g of the 8% protein hydrolysate. The fermentation broth exhibiting aminopeptidase activity was added until the relationship between enzyme and substrate (E/S) was 0.25%,

calculated on the basis of a product with 5000 LAPU/g (equivalent to 12.5 LAPU/g protein), and was then re-hydrolysed for 6 hours at 50°C, pH 7.0.

The pH and the osmolarity was determined (using standard methods) after 1 minute and 6 hours, respectively. % DH was determined using the TNSB-method (described above) and % FAA (% free amino acids) was determined using standard methods.

Aminopeptidase	pH after 1 minute	pH after 6 hours	Increase mOsm	% DH by TNBS-method	total % FAA
Transformant	6.47	6.65	59	7.01*	13
Blind	6.61	6.81	1		3

* the increase of DH caused by the hydrolysis of the substrate by the aminopeptidase.

DH of the blind is 28 %

A sample of debittered hydrolysate containing 13 % FFA was analyzed for the content of leucine. It was found that leucine constituted about 2.7%. while leucine constituted about 0.3% of the blind.

Example 12

Taste test

The taste of debittered protein hydrolysate was assessed by a tasting panel of 5 persons using a Bitterness Index (BI) between 0 (not bitter) and 10 (blind).

The bitterness, of a sample of bitter tasting 3.5 % protein hydrolysate (blind) and a similar sample subjected to the transformant, was assessed.

The Bitterness Index (BI) for the protein hydrolysate sample subjected to the transformant was found to be in the range of about 6.2.

This result shows that the 35 kDa aminopeptidase had debittered the protein hydrolysate samples.

Example 13

Bread flavour, dough stickiness and crumb structure

The flavour, dough stickiness and crumb structure of bread prepared using from 0 to 300 LAPU per kg flour were compared with bread prepared using the commercial product Flavourzyme®. The bread were prepared and assessed as described above in the "Material and Methods" section.

The following result (average of duplication) were found:

Enzyme(s)	LAPU/kg flour	Avg. vol. index	Flavour	Dough stickiness	Crumb structure
Flavourzyme®	80	112	4	3	crumby and open
Enzyme of the invention	0	100	3	4	as control
	10	100	3	4	as control
	30	100	3	4	as control
	50	100	3.25	4	as control
	80	98	3.5	4	as control
	100	102	3.5	4	as control
	150	97	4	4	as control
	200	99	4	4	as control
	300	100	4	4	as control

As can be seen from the above table the 35 kDa aminopeptidase of the invention gives a significant flavour enhancement in the form of a "fresh baked" bread smell when added in amounts from 30 to 300 LAPU per kg flour. The crumb structure is not affected by the aminopeptidase of the invention. The dough

stickiness is improved in comparison to the commercial protease/peptidase complex Flavourzyme®.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the claims below.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: An enzyme with aminopeptidase activity

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1409 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(B) STRAIN: *Aspergillus oryzae* A01568

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:52..1183

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CGAGCATTCC GTTCGTATCG ACTTGGTCGT ACACGCTTTC GTTCTCTCAA G ATG CGT      57
                                                          Met Arg
                                                            1

TTC CTC CCC TGC ATC GCG ACT TTG GCA GCC ACG GCC TCT GCC CTT GCT      105
Phe Leu Pro Cys Ile Ala Thr Leu Ala Ala Thr Ala Ser Ala Leu Ala
  5                  10                  15

ATT GGA GAC CAT GTA CGC TCG GAC GAT CAG TAT GTC CTA GAA CTC GCC      153
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Ile Gly Asp His Val Arg Ser Asp Asp Gln Tyr Val Leu Glu Leu Ala
     20                  25                  30

CCG GGA CAA ACG AAA GTT GTG ACG GAA GCA GAG AAA TGG GCT CTG AGA      201
Pro Gly Gln Thr Lys Val Val Thr Glu Ala Glu Lys Trp Ala Leu Arg
 35                  40                  45                  50

GCT GAG GGC AAG CGT TTC TTC GAT ATA ACC GAA CGG GCC AGT AGC CTG      249
Ala Glu Gly Lys Arg Phe Phe Asp Ile Thr Glu Arg Ala Ser Ser Leu
             55                  60                  65

GAA CTC GCA TCG AAC AAG AAA CAA AAG CTC GCG GTT ACC TAC CCC GAT      297
Glu Leu Ala Ser Asn Lys Lys Gln Lys Leu Ala Val Thr Tyr Pro Asp
         70                  75                  80

TCC GTG CAA CAC AAC GAG ACC GTG CAA AAT CTG ATC AAG TCG CTC GAC      345
Ser Val Gln His Asn Glu Thr Val Gln Asn Leu Ile Lys Ser Leu Asp
     85                  90                  95

AAA AAG AAC TTC GAA ACC GTT CTC CAG CCG TTC TCG GAG TTC CAC AAT      393
Lys Lys Asn Phe Glu Thr Val Leu Gln Pro Phe Ser Glu Phe His Asn
100                 105                 110

CGC TAT TAC AAG AGC GAC AAT GGC AAG AAA TCA TCC GAG TGG CTG CAA      441
Arg Tyr Tyr Lys Ser Asp Asn Gly Lys Lys Ser Ser Glu Trp Leu Gln
115                 120                 125                 130

GGC AAG ATT CAG GAA ATC ATC TCC GCC AGT GGA GCA AAG GGA GTC ACT      489
Gly Lys Ile Gln Glu Ile Ile Ser Ala Ser Gly Ala Lys Gly Val Thr
            135                 140                 145

GTG GAG CCT TTC AAA CAC TCC TTC CCG CAG TCG AGT CTG ATT GCG AAA      537
Val Glu Pro Phe Lys His Ser Phe Pro Gln Ser Ser Leu Ile Ala Lys
        150                 155                 160

ATC CCC GGC AAG AGT GAC AAG ACC ATC GTG CTT GGA GCG CAT CAG GAC      585
Ile Pro Gly Lys Ser Asp Lys Thr Ile Val Leu Gly Ala His Gln Asp
    165                 170                 175

TCC ATC AAC CTT GAT TCA CCC TCA GAG GGC CGT GCA CCG GGA GCT GAT      633
Ser Ile Asn Leu Asp Ser Pro Ser Glu Gly Arg Ala Pro Gly Ala Asp
180                 185                 190

GAC GAT GGA TCC GGC GTT GTT ACC ATT CTC GAA GCC TTC CGC GTT CTC      681
Asp Asp Gly Ser Gly Val Val Thr Ile Leu Glu Ala Phe Arg Val Leu
195                 200                 205                 210

CTG ACG GAC GAG AAG GTC GCA GCC GGT GAG GCT CCG AAC ACC GTT GAG      729
Leu Thr Asp Glu Lys Val Ala Ala Gly Glu Ala Pro Asn Thr Val Glu
            215                 220                 225

TTC CAC TTC TAT GCC GGA GAG GAG GGT GGT CTG CTG GGA AGT CAG GAC      777
Phe His Phe Tyr Ala Gly Glu Glu Gly Gly Leu Leu Gly Ser Gln Asp
        230                 235                 240
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ATC TTC GAG CAG TAC TCG CAG AAA AGC CGA GAC GTG AAA GCC ATG CTT      825
Ile Phe Glu Gln Tyr Ser Gln Lys Ser Arg Asp Val Lys Ala Met Leu
        245                 250                 255

CAA CAG GAT ATG ACG GGT TAT ACT AAA GGC ACA ACC GAT GCT GGA AAG      873
Gln Gln Asp Met Thr Gly Tyr Thr Lys Gly Thr Thr Asp Ala Gly Lys
    260                 265                 270

CCG GAG TCG ATC GGT ATC ATC ACT GAC AAT GTC GAT GAG AAC CTG ACC      921
Pro Glu Ser Ile Gly Ile Ile Thr Asp Asn Val Asp Glu Asn Leu Thr
275                 280                 285                 290

AAG TTC CTG AAG GTC ATT GTC GAT GCT TAT TGC ACT ATC CCG ACC GTC      969
Lys Phe Leu Lys Val Ile Val Asp Ala Tyr Cys Thr Ile Pro Thr Val
            295                 300                 305

GAT TCG AAA TGC GGA TAC GGA TGC TCT GAC CAT GCT TCT GCC ACG AAG      1017
Asp Ser Lys Cys Gly Tyr Gly Cys Ser Asp His Ala Ser Ala Thr Lys
        310                 315                 320

TAT GGT TAT CCC GCC GCA TTC GCA TTC GAG TCA GCC TTT GGC GAC GAC      1065
Tyr Gly Tyr Pro Ala Ala Phe Ala Phe Glu Ser Ala Phe Gly Asp Asp
    325                 330                 335

AGC CCT TAC ATT CAC TCG GCT GAT GAT ACG ATT GAG ACC GTC AAC TTT      1113
Ser Pro Tyr Ile His Ser Ala Asp Asp Thr Ile Glu Thr Val Asn Phe
340                 345                 350

GAC CAT GTG CTG CAA CAC GGC AAA CTG ACT CTT GGA TTT GCA TAT GAG      1161
Asp His Val Leu Gln His Gly Lys Leu Thr Leu Gly Phe Ala Tyr Glu
355                 360                 365                 370

CTT GCC TTC GCA GAT TCG CTG T AAGGCTTAAG ACGACGGTTG TATGAGCGAG      1213
Leu Ala Phe Ala Asp Ser Leu
            375

AGATCCAGTC CAACAGTGTG TATAAATAGT GGGCCCGTGT TCAAATAGCA CTTTGATTTA    1273

GCCAGTGAGT AGCTTTGGTG GCGAAAATGG AGCCCGAATT CTAGCCAACA TCGAACTGGA    1333

GCCTGTCAGG GCCCCATCAC AAGAAGTTTT GAGCTACATA AGCGAGATAA AAGTCAGAAA    1393

AAAAAAAAA AAAAA                                                      1409
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 377 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Arg Phe Leu Pro Cys Ile Ala Thr Leu Ala Ala Thr Ala Ser Ala
1 5 10 15

Leu Ala Ile Gly Asp His Val Arg Ser Asp Asp Gln Tyr Val Leu Glu
20 25 30

Leu Ala Pro Gly Gln Thr Lys Val Val Thr Glu Ala Glu Lys Trp Ala
35 40 45

Leu Arg Ala Glu Gly Lys Arg Phe Phe Asp Ile Thr Glu Arg Ala Ser
50 55 60

Ser Leu Glu Leu Ala Ser Asn Lys Lys Gln Lys Leu Ala Val Thr Tyr
65 70 75 80

Pro Asp Ser Val Gln His Asn Glu Thr Val Gln Asn Leu Ile Lys Ser
85 90 95

Leu Asp Lys Lys Asn Phe Glu Thr Val Leu Gln Pro Phe Ser Glu Phe
100 105 110

His Asn Arg Tyr Tyr Lys Ser Asp Asn Gly Lys Lys Ser Ser Glu Trp
115 120 125

Leu Gln Gly Lys Ile Gln Glu Ile Ile Ser Ala Ser Gly Ala Lys Gly
130 135 140

Val Thr Val Glu Pro Phe Lys His Ser Phe Pro Gln Ser Ser Leu Ile
145 150 155 160

Ala Lys Ile Pro Gly Lys Ser Asp Lys Thr Ile Val Leu Gly Ala His
165 170 175

Gln Asp Ser Ile Asn Leu Asp Ser Pro Ser Glu Gly Arg Ala Pro Gly
180 185 190

Ala Asp Asp Asp Gly Ser Gly Val Val Thr Ile Leu Glu Ala Phe Arg
195 200 205

Val Leu Leu Thr Asp Glu Lys Val Ala Ala Gly Glu Ala Pro Asn Thr
210 215 220

Val Glu Phe His Phe Tyr Ala Gly Glu Glu Gly Gly Leu Leu Gly Ser
225 230 235 240

Gln Asp Ile Phe Glu Gln Tyr Ser Gln Lys Ser Arg Asp Val Lys Ala
245 250 255

Met Leu Gln Gln Asp Met Thr Gly Tyr Thr Lys Gly Thr Thr Asp Ala
260 265 270

Gly Lys Pro Glu Ser Ile Gly Ile Ile Thr Asp Asn Val Asp Glu Asn
275 280 285

Leu Thr Lys Phe Leu Lys Val Ile Val Asp Ala Tyr Cys Thr Ile Pro
290 295 300

Thr Val Asp Ser Lys Cys Gly Tyr Gly Cys Ser Asp His Ala Ser Ala
305 310 315 320

Thr Lys Tyr Gly Tyr Pro Ala Ala Phe Ala Phe Glu Ser Ala Phe Gly
325 330 335

Asp Asp Ser Pro Tyr Ile His Ser Ala Asp Asp Thr Ile Glu Thr Val
340 345 350

Asn Phe Asp His Val Leu Gln His Gly Lys Leu Thr Leu Gly Phe Ala
355 360 365

Tyr Glu Leu Ala Phe Ala Asp Ser Leu
370 375

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Primer

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCIACRTIRT CIGIIATIAT YICIAT 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Primer

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GARACIGTIC ARAAYCTTAT 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Primer

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GAYAARAARA AYTTYGAWAC IGT 23

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Tyr Pro Asp Ser Val Gln His Xaa Glu Thr Val Gln Asn Leu Ile Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gly Val Thr Val Glu Pro Phe Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Val Ile Val Asp Ala Tyr Cys Thr Ile Pro Thr Val Asp Ser Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Gly Thr Thr Asp Ala Gly Lys Pro Glu Ser Ile Glu Ile Ile Thr Asp
1 5 10 15

Asn Val Asp Glu Asn Leu Thr Lys
20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Tyr Pro Asp Ser Val Gln His Xaa Glu Thr Val Gln Asn Leu Ile Lys
1 5 10 15

Ser Leu Asp Lys Lys Asn Phe Glu Thr Val Leu Gln Pro
20 25

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Asn Phe Glu Thr Val Leu Gln Pro Phe Ser Glu Phe His Asn Arg Tyr Tyr Lys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 12

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1272 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(B) STRAIN: *Aspergillus oryzae* A01568

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CTCGAGCAAT CCGTTCGTAT CGACTTGGTG GTACACGCTT TCGTTCTCTC AAGATCCGTT 60

TCCTCCCCTG CATCGCGACT TTCGCAGCCA CGGCCTCTGC CCTTGCTATT GGAGACCAIG 120

TACGCTCCGA CGATCAGTAT GTCCTAGAAC TCGCCCCCGG ACAAACGAAA GTTGTCACGG 180

AAGCAGAGAA ATGGGCTCTG AGACCTGAGG GCAAGCGTTT CTTCGATATA ACCGAACCGG 240

CCAGTAGCCT GGAACTCGCA TCGAACAAGA AACAAAAGCT CGCCGTTACC TACCCCGATT 300

CCGTCCAACA CAACGAGACC GTCCAAAATC TGATCAAGTC GCTCGACAAA AAGAACTTCG 360

AAACCGTTCT CCAGCCGTTC TCCGAGTTCC ACAATCGCTA TTACAAGAGC GACAATGCCA 420

AGAAATCATC CGAGTCGCTG CAAGCCAAGA TTCAGGAAAT CATCTCCGCC AGTCGAGCAA 480

ACCGAGTCAC TGTCGAGCCT TTCAAACACT CCTTCCCCGA GTCGAGTCTG ATTCCGAAAA 540

TCCCCCGCAA GAGTGACAAG ACCATCGTGC TTGGAGCCCA TCAGGACTCC ATCAACCTTG 600

ATTCACCCTC AGAGCCCCGT GCACCCCCAG CTGATGACGA TCGATCCCCC GTTGTTACCA 660

TTCTCGAAGC CTTCCCCGTT CTCCTGACCG ACGAGAAGGT CGCAGCCCGT GAGCCTCCGA 720

ACACCGTTGA GTTCCACTTC TATGCCCCAG AGGAGCGTCG TCTGCTCCCA AGTCAGGACA 780

TCTTCGAGCA GTACTCCCAG AAAACCCCAG ACGTGAAAGC CATGCTTCAA CAGCATATGA 840

CCCGTTTATAC TAAAGCCACA ACCGATGCTG GAAACCCCGA GTCGATCCGT ATCATCACTG 900

ACAATGTCGA TGAGAACCTG ACCAAGTTCC TGAAGCTCAT TGTCGATGCT TATTCCACTA 960

TCCCGACCGT CGATTCGAAA TCCCGATACG GATGCTCTGA CCATGCTTCT GCCACGAAGT 1020

ATGGTTATCC CGCCCCATTC GCATTCGAGT CAGCCTTTGG CGACGACAGC CCTTACATTC 1080

ACTCCGCCTA TGATACGATT GAGACCGTCA ACTTTGACCA TGTCCTGCAA CACGCCAAAC 1140

TGACTCTTCG ATTTGCATAT GAGCTTGCCT TCGCAGATTC GCTGTTAAGC TTATTGACGA C 1200

GGTTGTTATGA GCGAGAGATC CAGTCCAACA GTGTGTTATAA TATGTCGCCC CGTGTTCAAA 1260

TAGCACTTAA AA 1272

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 13, line 10-15

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany

Date of deposit	Accession Number
11.05.95	DSM No. 9965

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

During the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. e.g. Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71) in those designated states providing for such "expert solution".

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

PATENT CLAIMS

1. An enzyme exhibiting aminopeptidase activity derived from a fungal microorganism, which enzyme has an apparent molecular weight (M_w) of 35 kDa determined by SDS-page.

2. The enzyme according to claim 1, having an estimated isoelectric point (pI) in the range of about 4.9.

3. The enzyme according to claims 1 to 2, wherein the enzyme in the precursor-form has an estimated molecular weight of about 41 kDa.

4. The enzyme according to claim 3, wherein the enzyme in the precursor-form include a secretion signal.

5. The enzyme according to any of claims 1 to 4, derivable from a filamentous fungus or a yeast.

6. The enzyme according to claim 5, derivable from a filamentous fungus, such as *Aspergillus*, in particular *A. oryzae*, Especially *A. oryzae* A01568, or *Trichoderma*, *Pencillium*, *Fusarium* or *Humicola*.

7. The enzyme according to any of claims 1 to 6, which enzyme comprises or is comprised in the partial amino acid sequence shown in SEQ ID No. 6 or an analogue of said sequence, the partial amino acid sequence shown in SEQ ID No. 7 or an analogue of said sequence, and/or the partial amino acid sequence shown in SEQ ID No. 8 or an analogue of said sequence, and/or the partial amino acid sequence shown in SEQ ID No. 9 or an analogue of said sequence, and/or the partial amino acid sequence shown in SEQ ID No. 10 or an analogue of said sequence and/or the partial amino acid sequence shown in SEQ ID No. 11 or an analogue of said sequence.

8. The enzyme according to any of claims 1 to 7, which enzyme comprises or is comprised in the amino acid sequence shown in SEQ ID No. 2 or an analogue of said sequence.

9. The enzyme according to any of claims 1 to 8, which is immunologically reactive with an antibody raised against the purified aminopeptidase shown in SEQ ID No. 2 derived from *Aspergillus oryzae*, A01568.

10. The enzyme according to any of claims 1 to 9, which enzyme comprises in the range of 35 to 45% non-polar amino acids, preferably between 37 to 41%, and/or in the range of 30 to 40% polar amino acids, preferably between 33 to 37%, and/or in the range of 10 to 20% acidic amino acids, preferably between 12 to 16%.

11. The enzyme according to any of claims 1 to 10, which enzyme comprises in the range of 7 to 17% acidic amino acids, preferably between 10 to 14%.

12. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting aminopeptidase activity, which DNA sequence comprises

a) the aminopeptidase encoding part of the DNA sequence shown in SEQ ID No. 1, and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

b) an analogue of the DNA sequence shown defined in a), which

i) is homologous with the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965,

or

ii) hybridizes with the same oligonucleotide probe as the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 and/or the DNA sequence obtainable from *E. coli* DSM 9965, or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against the purified aminopeptidase encoded by the DNA sequence shown in SEQ ID No 1 derived from *Aspergillus oryzae* A01568 and/or obtainable from *E. coli*, DSM 9965.

13. The DNA construct according to claim 12, comprising the partial DNA sequences shown in SEQ ID No. 3, and/or the partial DNA sequences shown in SEQ ID No. 4, and/or the partial DNA sequences shown in SEQ ID No. 5.

14. The DNA construct according to any of claims 12 to 13, in which the DNA sequence is obtainable from a fungal microorganism, such as a filamentous fungus or a yeast.

15. The DNA construct according to claim 14, in which the DNA sequence is obtainable from a strain of *Aspergillus*, such as *Aspergillus oryzae*, in particular from the deposited *Aspergillus oryzae*, ATCC 20386, or *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola* or *E. coli* DMS 9965.

16. The DNA construct according to claim 30, in which the DNA sequence is isolated from or produced on the basis of a nucleic acid library of *Aspergillus oryzae*, A01568.

17. A recombinant expression vector comprising the DNA construct according to any of claims 12 to 16.

18. A cell comprising a DNA construct according to claims 12 to 16 or a recombinant expression vector according to claim 17.

19. The cell according to claim 18, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

20. The cell according to claim 19, wherein the cell belongs to a strain of *Aspergillus*, in particular a strain of *Aspergillus niger* or *Aspergillus oryzae*, a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp. *Pichia* sp., *Yarrowia* sp. such as *Yarrowia lipolytica*, or *Kluyveromyces* sp. such as *Kluyveromyces lactis*.

21. The cell according to claim 20, wherein the cell belongs to a strain of *Aspergillus*, in particular a strain of *Aspergillus niger* or *Aspergillus oryzae*.

22. An method for producing an enzyme exhibiting aminopeptidase activity, which method comprises cultivating a cell according to any of claims 18 to 21 in suitable culture medium under conditions permitting the expression of the DNA construct according to any of claims 12 to 16 or expression vector according to claim 17, and recovering the enzyme from the culture.

23. An enzyme preparation useful for reducing bitter taste of proteins or protein hydrolysates for foodstuff, which preparation is enriched with an enzyme exhibiting aminopeptidase activity of any of claims 1 to 11.

24. The enzyme preparation according to claim 23, which preparation is enriched with a factor of between 1.1 and 10, preferable between 2 and 8, especially between 4 and 6.

25. The enzyme preparation according to claims 23 and 24, which additionally comprises at least one other enzyme activity, including a cellulase, a hemicellulase, a pento-

sanase, a lipase, a peroxidase, an oxidase, a laccase, a xylanase, a peptidase, and an endo-protease.

26. A bread-improving or a dough-improving composition comprising an enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11.

27. The bread-improving or dough-improving composition according to claim 26 which further comprises an amylolytic enzyme, selected from the group comprising α-amylase, β-amylase, maltogenic α-amylase, acid stable amylase, 1,6-pullulanase and amyloglucosidase.

28. The bread-improving or dough-improving composition according to claim 26 and 27, which further comprises another enzyme such as a cellulase, a hemicellulase, a pentosanase, a lipase, a peroxidase, an oxidase, a laccase, and a xylanase.

29. The bread-improving or dough-improving composition according to any of claims 26 to 28, which further comprises another bread or dough improving agent.

30. A method of preparing a baked product from a flour dough, which method comprises, in the dough making process, to add an enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 to the dough or dough ingredients and subject the resulting dough to baking under suitable conditions.

31. The method of preparing a baked product from a flour dough according to claim 30, which method comprises, in the dough making process, further to add an amylolytic enzyme to the dough or dough ingredients and subject the resulting dough to baking under suitable conditions.

32. The method according to claim 31, in which the amylolytic enzyme is selected from the group consisting of α-amylase, β-

amylase, maltogenic α-amylase, acid stable amylase, 1,6-pullulanase, and amyloglucosidase.

33. The method according to any of claims 11-18, in which another enzyme such as a cellulase, a hemicellulase, a pentosanase, a lipase, a peroxidase, an oxidase, a laccase, an amylase, a xylanase is added to the dough or dough ingredients.

34. The method according to any of claims 30-34, in which the enzyme according to any of claims 1 to 11 is added in an amount corresponding 30 to 1000 LAPU, preferably 50 to 500 LAPU, especially 80 to 300 LAPU, such as about 100 LAPU per kg of flour.

35. The method according to any of claims 30-34, in which the enzyme(s) is/are added in the form of a bread-improving or dough-improving composition as defined in any of claims 26-29.

36. A method of preparing a frozen dough, which method comprises, in the dough making process, to add an enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 to the dough or dough ingredients and subject the resulting dough to freezing under suitable conditions.

37. The method according to claim 36, in which an amylolytic enzyme is added together with the enzyme exhibiting aminopeptidase activity according to claims 1 to 11.

38. The method according to any of claims 36 and 37, in which the enzyme(s) is/are added in the form of a bread-improving or dough-improving composition as defined in any of claims 26-29.

39. A baked product or a dough prepared by the method according to any of claims 30-38.

40. A pre-mix for dough comprising an enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or a bread-improving or dough-improving composition according to any of claims 26-29.

41. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or a composition according to any of claims 26 to 29 for improving the flavour of a baked product.

42. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or a composition according to any of claims 26 to 29 for improving the dough stickiness of a baked product.

43. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or a composition according to any of claims 26 to 29 for improving the crumb structure of a baked product.

44. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or a composition according to any of claims 26 to 29 for improving the crust colour of a baked product.

45. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or an enzyme preparation according to any of claims 23 to 25 for proteins or protein hydrolysates for foodstuff

46. The use according to claim 45, for debittering proteins or protein hydrolysates for foodstuff.

47. The use according to claims 45 and 46, wherein the protein or protein hydrolysate to be hydrolysed is of animal origin, such as casein or whey protein.

48. The use according to claim 47, wherein the foodstuff is cheese.

49. The use according to claims 45 and 46, wherein the protein or protein hydrolysate to be hydrolysed is of vegetable origin, such as soy protein.

50. The use according to claim 49, wherein the foodstuff comprises cocoa.

51. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or an enzyme preparation according to any of claims 23 to 25 for cleaning contact lenses.

52. Use of the enzyme exhibiting aminopeptidase activity according to any of claims 1 to 11 or an enzyme preparation according to any of claims 23 to 25 for brewing.

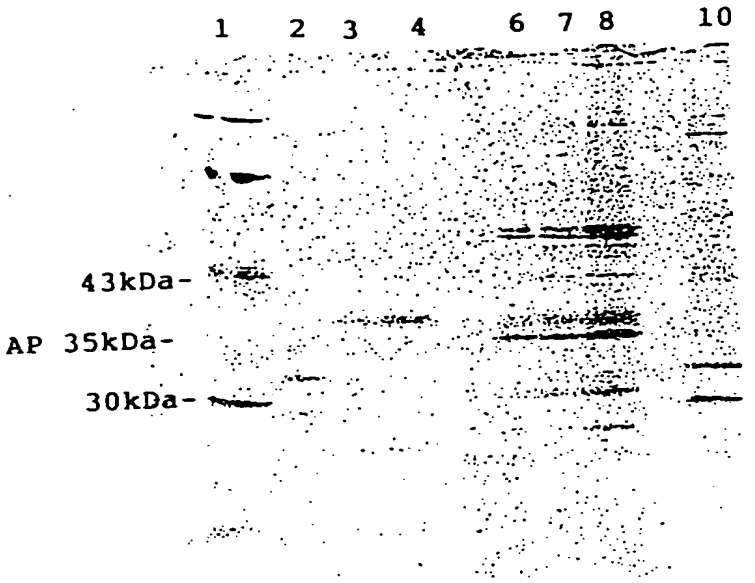

Figure 1

Lanes 1: Molecular weight marker.
Lane 2: Non-transformed *Aspergillus oryzae* A01560
Lanes 3 and 4: pA3EXP3/1 transformant (5 and 10 µl)
Lanes 6, 7 and 8: *Aspergillus oryzae* A01568
Lanes 10: A transformant using the same expression construct as for pA3EXP3/1 without aminopeptidase activity.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00104

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/48
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPI, WPIL, US PATENTS FULLTEXT, CA, SCISEARCH, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 5, BIOSIS, Dialog accession no. 8646033, Biosis no. 92111033, Matsuoka H et al: "Purification and Debittering Effect of Aminopeptidase II from Penicillium-Casei-colum"; & J Agric food Chem 39 (8). 1991. 1392-1395	1-52
X	WO 9426882 A1 (QUEST INTERNATIONAL B.V.), 24 November 1994 (24.11.94), page 12, line 4 - line 12; page 13, line 4 - line 27	1-52
X	US 5112812 A (ERNST-GUNNAR SAMUELSSON ET AL), 12 May 1992 (12.05.92), column 3, line 47 - line 54, claim 21	1-52

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 1996	04 -07- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00104

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0257821 A1 (GENENCOR INC.), 2 March 1988 (02.03.88), page 3, line 30, claim 1 --	51
A	Dialog Information Services, File 351, WPIL, Dialog accession no. 009834356, WPI accession no. 94-114212/14, KIKKOMAN CORP: "Prepn. of solid koji for brewing soy sauce - comprises using solid koji contg. specified zinc ion content per total wt of koji ingredient"; & JP,A,6062793 940308 9414 (Basic) -- --------	52

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00104

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 9, 12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see extra sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

The wording "immunologically reactive with an antibody raised against..." does not define a property that is relevant in the context of the invention, as there is no direct link between the aminopeptidase activity and the immunological features (except for some unknown epitopes of the acitve site). Due to this vague definition claims 9 and 12 does not fulfil the requirements of PCT article 6 regarding clarity and conciseness.

The wording "homologous" of claim 12 is not considered to be clear and concise since it has not been specified to what extent the sequence is homologous with the DNA sequence/polypeptide corresponding to SEQ ID No. 3 or the DNA sequence obtainable from E.coli DSM 9965 (c.f. PCT article 6). It should be clear from the claim that the part(s) that encodes the alleged inventive features of the aminopeptidase is present in the analogue which is defined "homologous".

The wording "hybridizes with the same oligoneucleotide probe" of claim 12 is not considered to be clear and concise since the part to which the oligonucleotide hybridizes with the analogue is not restricted to include the part(s) that encodes the alleged inventive features of the aminopeptidase (c.f. PCT article 6).

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.
PCT/DK 96/00104

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9426882	24/11/94	NONE	
US-A- 5112812	12/05/92	AU-B,B- 591230	30/11/89
		AU-A- 6834687	15/07/87
		AU-A- 6834787	15/07/87
		EP-A,A,B 0226221	24/06/87
		EP-A,A,B 0250501	07/01/88
		SE-T3- 0250501	
		FI-B- 89449	30/06/93
		IE-B- 59710	23/03/94
		JP-B- 8011039	07/02/96
		JP-T- 63502003	11/08/88
		JP-T- 63502004	11/08/88
		KR-B- 9403938	09/05/94
		WO-A,A- 8703785	02/07/87
		WO-A,A- 8703786	02/07/87
EP-A1- 0257821	02/03/88	AU-A- 7628087	04/02/88
		JP-A- 63158520	01/07/88
		US-A- 4749511	07/06/88

(19) BUNDESREPUBLIK DEUTSCHLAND

DEUTSCHES PATENTAMT

(12) **Offenlegungsschrift**

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C 12 N 1/15
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C 12 N 9/62
// (C12N 15/57,C12R 1:66) (C12N 1/15, C12R 1:66) (C12N 1/15,C12R 1:885)A23J 3/34

DE 195 26 485 A1

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(54) Rekombinant hergestellte Leucinaminopeptidase aus Aspergillus sojae

(57) Die Erfindung betrifft eine rekombinante Desoxyribonukleinsäure (DNA) isolierbar aus Aspergillus sojae, dadurch gekennzeichnet, daß sie für eine Leucin-Aminopeptidase (LAP) codiert und eine Nukleotidsequenz entsprechend der in SEQ ID NO:1 für die reife LAP angegebenen Nukleotidsequenz oder eine davon abgeleitete Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO:1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, aufweist. Die Erfindung betrifft weiterhin Vektoren, transformierte Wirtsorganismen sowie Verfahren zur Herstellung von LAP. Enzymprodukte zur Herstellung von Proteinhydrolysaten sowie entsprechend hergestellte Proteinhydrolysate sind ebenfalls Bestandteile der Erfindung.

DE 195 26 485 A1

Beschreibung

Gebiet der Erfindung

Die Erfindung betrifft eine rekombinante Desoxyribonukleinsäure (DNA) aus Aspergillus sojae, die für eine Leucin-Aminopepetidase (LAP) kodiert, Vektoren, die diese DNA sowie weitere DNA-Sequenzen zur Expression des LAP-Gens enthalten sowie mit diesen Vektoren transformierte filamentöse Pilze, die die rekombinante DNA exprimieren können. Weiterhin betrifft die Erfindung Enzym-Produkte, die eine mittels der rekombinanten filamentösen Pilze hergestellte rekombinante LAP enthalten sowie Verfahren zur Herstellung von Proteinhydrolysaten mit geringem Gehalt an Bitterstoffen mittels der rekombinanten LAP.

Stand der Technik

Enzymatisch erzeugte Proteinhydrolysate aus schwer verdaulichem oder schwer löslichem, tierischen oder pflanzlichen Einweiß, wie z. B. Gluten, Molke- oder Sojaproteinen, könnten in der Nahrungsmittelindustrie eine breite Anwendung z. B. als Zusätze für Aufschlagmassen, Babynahrung, Tierfutter, sowie allgemein für Fleisch- und Teigwaren finden.

Ein generelles Problem besteht darin, daß mit zunehmendem Hydrolysegrad der Einweiße Peptide entstehen, die einen unangenehmen bitteren Beigeschmack verursachen. Es sind schon zahlreiche Versuche unternommen worden, diesen bitteren Beigeschmack zu beseitigen. Keines der bisher entwickelten Verfahren konnte völlig befriedigen, so daß eine breite Anwendung bisher nicht stattfindet.

Nach neueren Erkenntnissen wird der Bittergeschmack durch einen Gehalt an Oligopeptiden verursacht, die bei fortschreitender Spaltung der nativen Proteine durch Endopeptidasen gebildet werden. Der Bittergeschmack tritt erst nach einem bestimmten Hydrolysegrad, bezogen auf die enthaltenen Peptidbindungen, auf. Dieser kritische Hydrolysegrad wird daher auch als Bitterpunkt bezeichnet. Der Bitterpunkt hängt stark vom hydrolysierten Protein ab. Ein Abbruch der Hydrolyse vor dem Erreichen des Bitterpunktes ist technisch schwer durchführbar und führt zudem dazu, daß die durch die Hydrolyse angestrebte bessere Verwertbarkeit nicht in vollem Umfang erreicht wird.

Die europäische Patentschrift EP 384 303 beschreibt ein Verfahren zur Herstellung von Proteinhydrolysaten mit niedrigem Gehalt an Bitterstoffen. Zur Hydrolyse werden eine Proteinase (Endopeptidase) und eine Aminopeptidase (Exopeptidase) aus einer Aspergillus-Kultur in einem ein- oder zweistufigen Verfahren verwendet. Als Quelle für das Peptidasegemisch werden toxikologisch unbedenkliche Stämme von Aspergillus-Arten wie z. B. A. oryzae, A. niger oder A. sojae genannt. Ein übermäßiges Entstehen von bitterschmeckenden Oligopeptiden wird dadurch vermieden, daß der Anteil an Endopeptidase gegenüber der Aminopeptidase begrenzt wird. Dies geschieht durch eine selektive thermische Inaktivierung der Endopeptidasen. Unter Anwendung des Verfahrens ist es möglich die Entstehung des Bitterpunktes zu verzögern und so vergleichsweise höhere Hydrolysegrade bei niedrigem Gehalt an Bitterstoffen zu verwirklichen.

Durch das genannte Verfahrens wird zwar eine Verbesserung erzielt, technisch wünschenswert wären jedoch weit höhere Proteolysegrade ohne gleichzeitige Entstehung von Bitterpeptiden. Ein weiterer Nachteil liegt darin, daß die thermische Inaktivierung der Endopeptidasen und somit die nachfolgende Proteinhydrolyse nicht völlig reproduzierbar ist. Dies gilt umsomehr wenn im einstufigen Verfahren nur eine teilweise Endopeptidase-Inaktivierung angestrebt wird. Die schlechte Reproduzierbarkeit kann ein Überschreiten des Bitterpunktes oder eine nicht ausreichende Hydrolyse zur Folge haben. Weiterhin ist anzuführen, daß der Aufwand des vorzunehmenden Inaktivierungsschrittes zu einer nicht unwesentlichen Verteuerung des eingesetzten Enzyms und damit des Verfahrens insgesamt beiträgt.

In den letzten Jahren sind eine Reihe von Genen für Pilzproteasen isoliert und exprimiert worden. WO 90100192 beschreibt die Isolierung des Gens für Aspergillopepsin A aus A. awamori. Beim Aspergillopepsin handelt es sich um eine Protease, die insbesondere bei der heterologen Genexpression in A. awamori zu einem proteolytischen Abbau des Fremdproteins führen kann. Bei der Expression des Kälber-Chymosins in A. awamori besteht weiterhin das Problem, daß das Aspergillopepsin unerwünschte Fehlgeschmäcke bei der Käseherstellung hervorrufen kann. Die genannte Patentanmeldung hat daher die Inaktivierung des unerwünschten Gens zum Ziel.

Die japanische Patentanmeldung JP 90-269370 beschreibt die Isolierung des Gens für alkalische Protease (1) aus einer chromosomalen Genbank von A. sojae. Diese alkalische Protease findet im südostasiatischen Raum eine breite Anwendung bei der Herstellung von Sojasoße. Die Herstellung von Sojasoße ist jedoch nicht Gegenstand der vorliegenden Erfindung.

Aufgabe und Lösung

Die Aufgabe der vorliegenden Erfindung war die Bereitstellung einer kostengünstigen Quelle für eine Endopeptidase für die Proteinhydrolyse, bei deren Verwendung die Entstehung des Bitterpunktes gegenüber dem jetzigen Stand der Technik nochmals weit verzögert wird, so daß die Prozeßsicherheit deutlich verbessert ist und zugleich die Kosten gesenkt werden. Die Aufgabe wird gelöst durch eine rekombinante Desoxyribonukleinsäure (DNA) isolierbar aus Aspergillus sojae, dadurch gekennzeichnet, daß sie für eine Leucin-Aminopeptidase (LAP) codiert und eine Nukleotidsequenz entsprechend der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz oder eine davon abgeleitete Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, aufweist.

Mittels dieser DNA können Vektoren, insbesondere Plasmide hergestellt werden mit denen Aspergillus-Stämme oder Trichoderma reesei-Stämme transformiert werden können. Aus den erhaltenen Transformanten können dann solche Stämme selektiert werden, die LAP in hohen Mengen exprimieren und sekretieren. Diese transformierten Wirtsorganismen erlauben wiederum Verfahren zur Herstellung der LAP in hohen Mengen. Aus den so gewonnenen Fermentationssäften können LAP-haltige Enzymprodukte hergestellt werden, die besonders gut zur Hydrolyse von Proteinen geeignet sind. Überraschenderweise wurde gefunden, daß eine Proteinhydrolyse mit den erfindungsgemäßen Enzymprodukten deutlich höhere Hydrolysegrade ohne Auftreten von Bittergeschmack ermöglicht als dies mit konventionell hergestellten Präparaten möglich ist.

Figuren, Sequenzbeschreibungen und Stammhinterlegungen

Fig. 1: Darstellung des Vektors pKD12

Sequenzbeschreibungen:
SEQ ID NO: 1: Chromosomales LAP-Gen aus A. sojae RH3782 SEQ ID NO: 2: Proteinsequenz der LAP aus A. sojae RH3782 mit Signalpeptidsequenz.
Stammhinterlegungen:
Folgende Mikroorganismen wurden bei der Deutschen Sammlung von Mikroorganismen (DSM) gemäß den Bestimmungen des Budapester Vertrages hinterlegt:

A sojae RH3782: Hinterlegungsnummer DSM 10090
E. coli DH5α pKD12: Hinterlegungsnummer DSM 10089
A. awamori RH3827: Hinterlegungsnummer DSM 10091

Ausführung der Erfindung

Rekombinante DNA

Die Erfindung betrifft eine rekombinante Desoxyribonukleinsäure (DNA) isolierbar aus Aspergillus sojae, dadurch gekennzeichnet, daß sie für eine Leucin-Aminopeptidase (LAP) codiert und eine Nukleotidsequenz entsprechend der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz oder eine davon abgeleiteten Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, aufweist.

Die Sequenzbeschreibung SEQ ID NO: 1 entspricht dem chromosomalen LAP-Gen aus A. sojae RH3782 mit 5'- und 3'-flankierenden Sequenzen. Unter der Nukleotidsequenz für die reife LAP wird die in SEQ ID NO: 1 enthaltene, für das Strukturgen der LAP ohne das Signalpeptid codierende DNA-Sequenz verstanden. Die Nukleotidsequenz für die reife LAP sind somit diejenigen Exon-Sequenzen, die für die Aminosäuren 1 (Tyr) bis 298 (Leu) codieren.

Die Erfindung betrifft weiterhin eine von der Nukleotidsequenz für die reife LAP abgeleitete Nukleotidsequenz. Darunter sind Nukleotidsequenzen zu verstehen, die von der in SEQ ID NO: 1 angegebenen Nukleotidsequenz abweichen, aber unter stringenten Bedingungen mit der DNA für die LAP hybridisieren.

Der Begriff "stringente Hybridisierungsbedingungen" ist dem Fachmann geläufig (Siehe z. B. Maniatis et al. (1982): Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Stringente Hybridisierungsbedingungen sind solche, unter denen nur DNA-Moleküle mit einem hohen Homologiegrad, z. B. >85%, miteinander hybridisieren. Die Stringenz der Versuchsbedingungen kann z. B. durch die Hybridisierungstemperatur oder durch die Salzkonzentration der Hybridisierungslösung eingestellt werden.

Vom LAP Strukturgen abgeleitete DNA-Sequenzen können z. B. Abweichungen in der Nukleotidsequenz ohne Abweichungen in der Aminosäuresequenz, bedingt durch die Degeneration des genetischen Codes, aufweisen. Ebenso können geringfügige Abweichungen in der Nukleotidsequenz auch zu funktionell unwesentlichen Änderungen in der Aminosäuresequenz des Enzyms führen. Weitgehend homologe Gene die mittels der vorliegenden Erfindung aus anderen Stämmen von A. sojae oder nah verwandten Aspergillus-Arten isoliert werden können sind daher eingeschlossen. Von der Anspruchsdefinition sind auch Fusionsproteine eingeschlossen, die für die enzymatische Funktion des Proteins wesentliche Teile der Teile des LAP-Gens bzw. davon abgeleitete Nukleotidsequenzen aufweisen.

Die Sequenzbeschreibung SEQ ID NO: 1 enthält weiterhin die vor der Nukleotidsequenz für die reife LAP liegende, codierende Sequenz für das Signalpeptid, beginnend mit der Aminosäure −47 (Met) bis -1 (Thr). Vor der Signalpeptidsequenz (Nukleotid 1−581) liegt die funktionelle Promotorregion des LAP-Gens. Hinter dem Strukturgen befindet sich das TAA-Stopcodon sowie eine als Transkriptionsterminator funktionelle Region (Nukleotide 1742−1873). Die Sequenzbeschreibung SEQ ID NO: 2 gibt die gesamte Aminosäuresequenz der LAP mit dem Signalpeptid wieder.

Die erfindungsgemäße rekombinante DNA kann durch die Isolierung eines LAP-Gens aus einem A. sojae, z. B. aus A. sojae RH3782, erhalten werden. Dazu kann die LAP aus einem Kulturfiltrat des Stammes gereinigt werden. Dies ist ein kritischer Arbeitsschritt, da eine Reinigungsmethode zunächst entwickelt werden muß. Die LAP kann durch Ionenaustauschchromatographie und Gelfiltration von den übrigen Proteinen abgetrennt werden. Hier muß ein Reinheitsgrad erreicht werden, der eine eindeutige Sequenzierung des N-Terminus des Proteins oder von Peptiden daraus ermöglicht. Von den Proteinfragmenten lassen sich in an sich bekannter Weise durch Rückübersetzung des genetischen Codes DNA-Sonden ableiten. Derartige DNA-Sonden können nach Vorlage im Handel bestellt werden oder können mit Hilfe eines DNA-Synthesizers in bekannter Weise

selbst hergestellt werden.

Die beanspruchte DNA kann aus dem Genom eines LAP produzierenden Aspergillus-Stammes, insbesondere eines A. sojae-Stammes erhalten werden. Dazu wird RNA und/oder DNA aus dem Zellmaterial des Aspergillus-Stammes gewonnen. Daraus können mit Hilfe geeigneter Vektoren Copy DNA(cDNA)- und/oder genomische Genbanken angelegt werden. Für eine genomische Genbank kann z. B. der Phage λ EMBL3 als Vektor verwendet werden. Eine cDNA-Genbank kann z. B. in E. coli DH5α mit dem Plasmid pUC 18 angelegt werden.

Es ist günstig für die Anlage der cDNA-Bank, das Ausgangszellmaterial in einem Medium anzuziehen, in dem der Stamm möglichst viel LAP produziert, da in diesem induzierten Zellmaterial der Gehalt an LAP-spezifischer mRNA höher als in nicht induziertem Zellmaterial ist. Dadurch kann ein höherer Anteil an LAP-spezifischen Klonen in der cDNA-Genbank erhalten werden. Geeignete Anzuchtmedien sind z. B. Minimalmedien für Pilze, denen eine proteinreiche Hauptstickstoffquelle, z. B. Milchpulver oder Pepton, zugesetzt wird. Bei maximalem LAP-Titer im Medium kann die Zellernte vorteilhaft durch das Einfrieren des abfiltrierten Mycels in flüssigem Stickstoff erfolgen.

Aus dem Zellmaterial kann die Präparation von polyadenylierter mRNA in an sich bekannter Weise erfolgen. Dazu kann das Mycel z. B. in Gegenwart von Detergentien und RNase-Inhibitoren wie z. B. Heparin, Guanidinium-Isocyanat oder Mercaptoethanol, homogenisiert und die mRNA durch Phenol-, Phenol-Chloroform- ggf. mit Zusatz von Isoamylalkohol, Chloroform- und/oder Diethlyether-Extraktionen gewonnen werden. Optional können die Extraktionsmischungen zusätzlich Salze oder Puffer oder Kationen-chelierende Agenzien in bekannter Weise enthalten. Die mRNA kann anschließend aus der wäßrigen Phase durch Zusatz von Ethanol präzipitiert werden oder durch chromatographische Methoden, z. B. Affinitäts-Chromatographie an Oligo(dT)- oder Oligo(dU)-Sepharose, gewonnen werden. Ebenso kann eine Gradientenzentrifugation, z. B. in einem linearen Saccharosegradienten, oder eine Agarosegelchromatographie zur weiteren Reinigung der isolierten mRNA verwendet werden.

Aus der mRNA kann in an sich bekannter Weise mittels reverser Transkriptase (RNA abhängige DNA-Polymerase) zunächst eine komplementäre einzelsträngige DNA und dann eine doppelsträngige cDNA mit Hilfe einer DNA-abhängigen DNA-Polymerase erzeugt werden. Dazu wird die mRNA mit einer Mischung von Desoxynucleosidtriphosphaten (dATP, dCTP, dGTP und dTTP), die optional radioaktiv markiert sind, um das Resultat der Reaktion nachvollziehen zu können, und einem Primer-Oligonukleotid, z. B. einem Oligo-dT-nukleotid, das mit dem Poly-A-Ende der mRNA hybridisiert, und einer reversen Transkriptase, z. B. der reversen Avian Myoblastosis Virus (AMV) Transkriptase, inkubiert, die eine komplementäre einzelsträngige DNA erzeugt. In einem zweiten Schritt kann dann die einzelsträngige DNA z. B. mit der DNA-Polymerase 1 wiederum komplementär zu einer doppelsträngigen cDNA ergänzt werden. Die cDNA kann mit Hilfe von DNA-Linkern in einen Vektor, z. B. den Phagen λgt10 inseriert werden.

Die erhaltenen Phagenklone können auf einem Agar mit E.coli-Wirtszellen vermehrt werden. DNA von diesen Agarplatten kann auf Nitrocellulose-Filter übertragen werden, welche mit einer z. B. radioaktiv markierten DNA-Sonde hybridisiert werden. Von den Nitrocellulosefiltern können dann Autoradiographien angefertigt werden aus denen die Position von Klonen, die das gesuchte LAP-Gen oder Teile davon enthalten auf den Agarplatten abgeleitet werden können. Auf diese Weise können entsprechende cDNA-Phagenklone aus der Genbank isoliert werden.

Genomische DNA kann unabhängig von den Anzuchtbedingungen der Zellen aus dem Mycel gewonnen werden. Bevorzugt ist eine Anzucht des Zellmaterials in einem Vollmedium für Pilze, z. B. Sabouraud-Bouillion. Die Isolierung der DNA aus dem Zellmaterial kann in an sich bekannter Weise durch Homogenisierung des Zellmaterials in einem Puffer, z. B. 100 mM Tris/HCl pH 8,0, und anschließende Phenol-, Phenol/Chloroform-Extraktion erfolgen. Die DNA kann durch Zusatz von Ethanol aus der wäßrigen Phase präzipitiert werden und ggf. weiter, z. B. durch CsCl-Dichtegradientenzentrifugation, gereinigt werden.

Die DNA kann dann partiell mit einen Restriktionsenzym, z. B. Sau3A, geschnitten werden und mit einem Vektor, z. B. λEMBL3, ligiert werden. Die erhaltenen Phagenklone können auf einem Agar mit E.coli-Wirtszellen vermehrt werden. DNA von diesen Agarplatten kann auf Nitrocellulose-Filter übertragen werden, welche mit einer z. B. radioaktiv markierten DNA-Sonde hybridisiert werden. Von den Nitrocellulosefiltern können dann Autoradiographien angefertigt werden aus denen die Position von Klonen, die das gesuchte LAP-Gen oder Teile davon enthalten auf den Agarplatten abgeleitet werden können. Auf diese Weise können entsprechende Phagenklone aus der Genbank isoliert werden.

Diese DNA-Sonden können zum Screening in Genbanken verwendet werden. Günstig ist es zunächst eine aus der mRNA des Wirtsstammes hergestellte cDNA in einer cDNA-Genbank zu screenen. Mit Hilfe der spezifischen cDNA als Probe läßt sich dann auch das chromosomale Gen aus der genomischen Genbank auffinden. Nach dem Auffinden eines spezifischen Phagen-Klons, erfolgt üblicherweise eine Subklonierung in ein Plasmid, z. B. pBR322, pUG18 oder pUC19.

Die Charakterisierung der isolierten DNA erfolgt in an sich bekannter Weise durch Restriktionsanalyse und anschließende Sequenzierung, z. B. nach der Sanger-Methode. Durch den Vergleich der cDNA Sequenz mit der Sequenz des chromosomalen Gens kann die Lage der Exon- und Intronsequenzen bestimmt werden. Die Signalpeptidsequenz liegt zwischen dem ATG-Startcodon der kodierenden Sequenz und dem Beginn des reifen Protein, das durch die Übereinstimmung mit der ermittelten N-terminalen Aminosäuresequenz bestimmt werden kann.

Vektoren zur Expression der LAP in Aspergillus- oder T. reesei-Stämmen

Die Erfindung betrifft einen Vektor enthaltend

a) DNA-Sequenzen zur Replikation des Vektors in E. coli,
b) DNA-Sequenzen zur Expression und Sekretion eines Polypeptids in einem Aspergillus-Stamm oder in einem Trichoderma reesei-Stamm, die für einen Promotor, eine Signalpeptidsequenz und optional für einen Terminator codieren,
c) eine für ein Polypeptid codierende DNA-Sequenz, die funktionell mit den DNA-Sequenzen nach b) verbunden ist,

dadurch gekennzeichnet, daß
die DNA-Sequenz nach c) eine Nukleotidsequenz entsprechend der in SEQ ID NO: 1 für die reife LAP angegeben Nukleotidsequenz oder eine davon abgeleitete Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, aufweist.

Die DNA-Sequenzen nach a) werden benötigt, um die Vektor-DNA in E. coli, z. B. E. coli DH5α, vermehren zu können. Eine derartige DNA-Sequenz kann ein Phage, z. B. der Phage λEMBL3, ein Cosmid oder bevorzugterweise ein Plasmid sein. Geeignete Plasmide sind z. B. pBR322, pUC18 oder pUC19 oder ggf. Fragmente dieser Plasmide, die zumindest den "origin of replication" und einen Selektionsmarker für E. coli enthalten.

Die Vektoren enthalten weiterhin DNA-Sequenzen nach b), die in Aspergillus-Stämmen oder in T. reesei-Stämmen zur Expression und Sekretion des Gens des LAP-Gens nach c) führen. Diese DNA-Sequenzen nach b) sind mit dem LAP-Gen funktionell verbunden. Dies sind z. B. 5'- und 3'-flankierende Sequenzen wie 5' vor dem Gen angeordnete Promotoren, eine Signalpeptidsequenz, 3' hinter dem Gen angeordnete Terminatoren. Weitere funktionelle DNA-Sequenzen, die vorhanden sein können sind z. B. Ribosomenbindungsstellen für die Translation, Enhancer oder "Upstream Activating Sequences" oder Polyadenylierungsfunktionen.

Signalpeptidsequenzen sind 5' unmittelbar vor dem Strukturgen liegende, für Aminosäuren codierende DNA-Sequenzen, die bei extrazellulären Proteinen vorkommen und gemeinsam mit dem Strukturgen transkribiert und translatiert werden. Bei der Sekretion des Proteins aus der Zelle werden die Signalpeptidsequenzen abgespalten wodurch das eigentliche "reife" Protein entsteht. Ein Signalpeptidsequenz ist somit notwendig um die Sekretion der LAP aus der Wirtszelle zu erreichen. Ebenso sind ein Promotor und ein Terminator zur Initiation der Translation bzw. zur Termination der Transkription des Gens notwendig.

Es kann sich dabei um den natürlich im Chromosom von A. sojae liegenden Promotor, die Signalpeptidsequenz und/oder den Terminator des LAP-Gens handeln. Ebenso können die funktionellen DNA-Sequenzen von anderen Genen stammen, die in Pilzstämmen der Gattung Aspergillus oder in T. reesei exprimiert und sekretiert werden.

Beispiele für Gene die geeignete funktionelle DNA-Sequenzen aufweisen sind z. B. das TAKA-Amylase A Gen aus A. oryzae (EP 238 023), das Pektinesterase-Gen oder das Polygalakturonidase-Gen aus A. niger (EP 388 593), das Glucoamylase-Gen aus A. awamori (EP 215 594) das Cellobiohydrolase-Gen I (cbh 1) aus T. reesei (EP 244234).

Die DNA-Sequenz nach c) enthält das LAP-Strukturgen oder eine davon abgeleitete DNA-Sequenz. Diese DNA-Sequenz kann z. B. in Form eines chromosomalen Gens mit den natürlich enthaltenen Introns oder als von der Messenger Ribonukleinsäure (mRNA) abgeleitete cDNA ohne Introns enthalten sein.

Ein erfindungsgemäßer Vektor ist z. B. das Plasmid pkD12 (siehe **Fig. 1**). Das Plasmid besteht aus dem allgemein bekannten E. Coli Plasmid pUC19 sowie einem Hind/III/EcoRI-Restriktionsfragment aus der chromosomalen DNA von A. sojae RH3782. Das Hind/III/EcoRI-Restriktionsfragment enthält das Strukturgen der LAP mit der natürlichen, für das Signalpeptid kodierenden DNA-Sequenz sowie den natürlichen, 5' vor dem Gen liegenden Promotor und 3' hinter dem Gen liegenden Terminationssequenzen. Die vor und hinter dem Strukturgen einschließlich der Signalpeptidsequenz liegenden DNA-Sequenzen sind funktionell und führen zur Expression und Sekretion in filamentösen Pilzen der Gattung Aspergillus, z. B. in Stämmen von A. sojae oder A. oryzae.

Dem Fachmann sind hinreichend Methoden bekannt, mit deren Hilfe ein Gen mit funktionellen DNA Sequenzen, z. B. einem anderen Promotor oder einer Signalpeptidsequenz, in einem Vektor kombiniert werden kann (Siehe z. B. Maniatis et al. (1982): Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Die Nukleotidsequenz für das reife LAP Gen oder eine davon abgeleitete Nukleotidsequenz kann daher auch mit anderen funktionellen Sequenzen als den in pKD12 vorhandenen funktionellen DNA-Sequenzen verbunden werden, die zur Expression und Sekretion der LAP in einem filamentösen Pilz der Gattung Aspergillus oder in T. reesei geeignet sind.

Von besonderer Bedeutung in Bezug auf die Höhe der Expression sind vor allem funktionelle Promotorsequenzen. Hierbei handelt es sich um DNA-Sequenzen von ca. 500—2000 Basenpaaren Länge, die jeweils 5' vor dem Startcodon eines Aspergillus- oder eines T. reesei-Gens liegen.

Solche DNA-Sequenzen können z. B. als Restriktionsfragmente isoliert werden und mit Restriktionsfragmenten des LAP-Gens einschließlich der Signalpeptidsequenz ligiert werden. Dabei können nicht kompatible Restriktionsschnittstellen oder Bereiche ohne geeignete Restriktionsschnittstellen z. B. durch synthetisch hergestellte Oligonukleotide überbrückt bzw. ersetzt werden, so daß die ursprüngliche DNA-Sequenz erhalten bleibt. Auf diese Weise kann die DNA-Sequenz des LAP-Gens einschließlich des Signalpeptids unverändert erhalten bleiben und mit einer ebenfalls unveränderten Promotorsequenz funktionell verbunden werden.

Zur Erhöhung der Expression kann der natürliche Promotor gegen einen anderen ausgetauscht werden. Es sind zahlreiche geeignete Promotoren bekannt. Geeignet ist z. B. der TAKA-Amylase Promotor aus A. oryzae (siehe EP 238 023) zur Expression in A. oryzae- oder A. sojae-Stämmen, der gpdA-Promotor aus A. nidulans (PUNT et al. (1987) Gene 56, S. 117 — 124) zur Expression in A. nidulans-, A. niger-, A. phoenicis, A. japonicus, A. foetidus oder A. awamori-Stämmen. Geeignet für die Expression in einem T. reesei-Stamm ist z. B. der cbh1-Promotor aus T. reesei (EP-A-244234).

Als Terminator wird die natürliche hinter dem Strukturgen liegende chromosomale Terminator-Sequenz

gemäß SEQ. ID NO: 1 bevorzugt. Je nach dem zur Expression verwendeten Stamm oder der verwendeten Promotorsequenz kann es von Vorteil sein zusätzlich die natürliche Leadersequenz oder auch die Terminationssequenz auszutauschen, um eine nochmals verbesserte Expression und Sekretion zu erreichen.

Geeignet ist z. B. der trpC Terminator aus A. nidulans (PUNT et al, s. o.) oder der Pektinesterase-Terminator aus A. niger (EP 388 593).

Transformierte Aspergillus- oder Trichoderma-Stämme zur großtechnischen Produktion von LAP

Die erfindungsgemäßen Plasmide können bei der Transformation von Aspergillus- oder Trichoderma reesei-Stämmen verwendet werden. Die Plasmide können dabei mehrfach ins Genom der Wirtsstämme integriert werden. Durch die Erhöhung der Anzahl der Genkopien und/oder die zusätzliche Verwendung stärkerer Promotoren kann die LAP-Produktivität bedeutend erhöht werden. Aus einer Vielzahl von Transformanten können solche mit besonders hoher Produktivität ausgewählt werden. Durch die Förderung der LAP-Produktivität treten zugleich Nebenaktivitäten, wie eine unerwünschte Endoproteinase-Aktivität, in den Hintergrund. Erfindungsgemäß transformierte Stämme eignen sich daher besonders gut zur großtechnischen Produktion von LAP-Enzymprodukten.

Besonders geeignet zur Expression und Sekretion der LAP sind Pilz-Stämme von Arten, deren gute Produktionseigenschaften für Enzyme bekannt sind. Bevorzugt sind insbesondere Stämme der Arten A. niger, A. awamori, A. phoenicis, A. japonicus, A. foetidus, A. oryzae oder A. sojae, sowie T. reesei. Geeignete Stämme können aus öffentlich zugänglichen Stammsammlungen wie z. B. ATCC, DSM, CBS oder NRRL erhalten werden. Geeignete Wirtsstämme sind z. B. A. awamori ATCC 11360, A. foetidus ATCC 10254 und ATCC 1035, A. japonicus 16873, A. oryzae NRRL 695, A. niger ATCC 10864, A. phoenicis CBS 136.52 sowie T. reesei ATCC 26921. Besonders geeignet ist A. awamori NRRL 3112. Ebenfalls besonders geeignet ist der Gendonorstamm A. sojae RH3782 (hinterlegt bei DSM).

Die Transformation der Pilzstämme kann mit Hilfe bekannter Methoden vorgenommen werden. EP 184438 (US 4 885249) beschreibt z. B. eine Transformationsmethode für A. niger, bei der als Selektionsmarker das argB-Gen aus A. nidulans verwendet wird. EP 238 023 beschreibt im Beispiel 9 eine generell anwendbare Transformationsmethode für A. oryzae-Stämme. Dabei wird das Plasmid p3SR2 mit dem amdS-Gen aus A. nidulans als Selektionsmarker verwendet. EP 244234 beschreibt die Transformation von T. reesei mit diesem Vektor. Die Transformation von A. niger mit p3SR2 wird von KELLY und HYNES (1985), EMBO Journal 4, S. 475—479, beschrieben. Für Stämme der Arten A. niger, A. awamori, A. japonicus, A. phoenicis und A. foetidus kann bevorzugt der Vektor pAN7-1 (PUNT et al. (1987) Gene 56, S. 117—124) verwendet werden. Transformanten können dann anhand der Resistenz gegen Hygromycin B selektiert werden.

Zur Transformation werden zunächst aus vegetativen Zellen oder auch aus Konidiosporen unter Enzymeinwirkung Protoplasten in einem osmotisch stabilisiertem Medium erzeugt. Zu diesen werden üblicherweise nach mehreren Waschschritten die zu transformierende Plasmid-DNA in Gegenwart von Polyethylenglykol (PEG) und $CaCl_2$ zugegeben, was zur Aufnahme der DNA in die Zellen führt. Nach der Transformation werden die Protoplasten auf osmotisch stabilisiertem Medium regeneriert, wobei eine Selektion auf solche Zellen erfolgt die ein Markergen aufgenommen haben.

Die Transformation der Pilzstämme erfolgt bevorzugt nach dem Co-Transformationsverfahren bei dem ein Plasmid mit einem Selektionsmarker für Aspergillus- oder T. reesei-Stämme und ein erfindungsgemäßer Vektor — eine Phagen-DNA oder eine Cosmid-DNA oder bevorzugt ein Plasmid mit dem LAP-Gen — gleichzeitig zu den zu transformierenden Zellen zugegeben werden.

A. awamori NRRL 3112 kann beispielsweise mit den Plasmiden pAN7-1 und pKD12 cotransformiert werden. Es können dann gegen Hygromycin B resistente Transformanten selektiert werden, die in Schüttelkolben auf die Produktion von LAP geprüft werden können. Eine Beispiel für eine auf diese Weise erhaltene, LAP-exprimierende Transformante von A. awamori NRRL 3112 ist der Stamm A. awamori RH3827, der bei DSM hinterlegt wurde (siehe oben unter Stammhinterlegungen).

Gut geeignet ist z. B. das amdS-Gen aus A. nidulans, das z. B. auf dem Vektor p3SR2 vorhanden ist. Mit diesem Plasmid transformierte Aspergillus oder Trichoderma-Stämme, die vorher schlecht auf einem Minimalnährboden mit dem künstlichen Substrat Acetamid als alleiniger Stickstoffquelle wuchsen, können nach der Transformation mittels eines deutlichen Wachstumsvorteils selektiert werden. Unter den insgesamt erhaltenen Transformanten müssen dann solche mit erhöhter oder besonders hoher LAP-Produktivität ausgewählt werden. Diese Auswahl kann z. B. aufgrund der LAP-Produktivität der Transformanten in Schüttelkolben getroffen werden.

Verfahren zur Herstellung von LAP mit einem erfindungsgemäß transformierten Wirtsorganismus

Der transformierte Wirtsstamm kann in einem geeigneten Medium in Submerskultur inkubiert werden. Geeignete Medien sind solche in denen filamentöse Pilze ein gutes Wachstum zeigen, insbesondere solche in denen zugleich eine gute Bildung der LAP erfolgt. Gut geeignet sind Medien von denen bekannt ist, daß der jeweilige Aspergillus- oder T. reesei-Wirtsstamm ein gutes Wachstum zeigt und zugleich LAP bildet. Besonders günstig auch im Hinblick auf kostengünstige Medien ist die Verwendung billiger Naturprodukte als Nährbodenbestandteile, wie z. B. Maisquellpulver oder Maisquellwasser, Roggenkleie, Weizenkleie, Kartoffeldextrin, Maltodextrin, Kartoffelprotein, Melasse etc. Günstig ist ebenfalls die Verwendung von Ammoniumsalzen als N-Quellen, z. B. Ammoniumsulfat. Der Fachmann kann sich an für die Fermentation von Aspergillus- oder Trichoderma reesei-Stämmen bekannten Medien orientieren und durch Versuche besonders geeignete Medien herausfinden. Ein geeignetes Medium kann z. B. 5% Maltodextrin und 5% Weizenkleie in Leitungswasser enthalten.

Die Herstellung der LAP kann durch Submersfermentation erfolgen. Die Beimpfung kann dabei üblicherwei-

se über eine Impfkaskade vom Kulturröhrchen oder einer Petrischalenkultur über Schüttelkolben ggf. einen oder mehrere Vorfermenter in einen Hauptfermenter erfolgen. Eine übliche Fermentationsdauer beträgt ca. 30 bis 70 Stunden bei ca. 28° Celsius unter aeroben Bedingungen. Nach Abbruch der Fermentation wird das Zellmaterial, z. B. durch Filtration,- abgetrennt und der LAP-haltige Kultursaft geerntet. Der Kultursaft kann zu flüssigen oder trockenen LAP-Produkten, z. B. durch Sprühtrocknung oder Sprühgranulation, weiterverarbeitet werden. Auf diese Weise können LAP-Enzymprodukte hergestellt werden, die zur Hydrolyse von Proteinen geeignet sind.

Proteinhydrolyse unter Verwendung eines erfindungsgemäßen LAP-Enzymproduktes

Die erfindungsgemäß auf rekombinantem Wege hergestellte LAP eignet sich hervorragend zur Hydrolyse von zahlreichen tierischen oder pflanzlichen Einweißstoffen zu wertvollen Proteinhydrolysaten, die geschmacksneutral oder wohlschmeckend und im wesentlichen frei von Bittergeschmack sind. Beispiele hydrolysierbarer Proteine sind Soja-Protein, Gluten, Getreide- und Bohnenproteine, Kartoffelprotein, Hefeprotein und andere mikrobielle Proteine, Hämoglobin und Fleisch- und Fischproteinmaterial. Vorzugsweise wird das Verfahren für die Hydrolyse von Milchproteinen, insbesondere Casein und Molkenprotein eingesetzt.

Die Hydrolyse wird am besten im Temperaturbereich von 10 bis 60 C, vorzugsweise von 25 bis 45°C, in 1 bis 10 Stunden unter Rühren durchgeführt. Zweckmäßig werden die Ausgangsproteine in einer Aufschlämmung oder Lösung mit einem Festgehalt von 5 bis 15 Gew.-% eingesetzt. Der pH-Wert liegt im allgemeinen im Bereich von 5 bis 9,5, vorzugsweise bei 6 bis 8.

Das Verfahren kann ein- oder zweistufig ausgeführt werden. Vorzugsweise wird das Verfahren einstufig durchgeführt indem man eine handelsübliche Endo-Proteinase in geringer Konzentration und die LAP gleichzeitig einwirken läßt. Zweckmäßig sind pro 100 g zu hydrolysierendem Protein 0,01 bis 10 Ansoneinheiten Endo-Proteinase und 10^5 bis 10^7 Einheiten an LAP. Dabei können unter Verwendung der rekombinanten LAP wesentlich höhere Hydrolysegrade ohne die Entstehung von Bitterpeptiden erzielt werden als dies etwa mit der in EP 384 303 beschriebenen konventionellen LAP möglich ist.

Wenn der erwünschte Hydrolysegrad erreicht ist, werden die Endo-Proteinase und die LAP inaktiviert, zweckmäßig durch kurzzeitiges Erhitzen auf ca. 100° C. Alternativ ist es auch möglich eine Inaktivierung durch Ansäuerung auf pH 3—4 zu erreichen. Ggf. kann auch eine Kombination beider Methoden angewendet werden. Das Proteinhydrolysat kann in gewünschter Weise zu Nahrungs- oder Futtermitteln verarbeitet werden. Es kann z. B. in flüssiger Form oder nach Sprüh- oder Walzentrocknung weiterverarbeitet werden.

Beispiele

(Prozentangaben in den Beispielen bedeuten Gew.-% soweit nicht anders angegeben)

Beispiel 1

Reinigung der LAP aus A. sojae

100 ml Kulturfiltrat von A. sojae RH3782 wurden mit destilliertem H_2O auf 1000 ml aufgefüllt und anschließend durch Zentrifugation für 5 min bei 5000 g und 25° Celsius geklärt. Um die elektrische Leitfähigkeit herabzusetzen wurde die Probe über eine Sephadex G25-Säule (MERCK) entsalzt. Die aufgefangene Enzymlösung hatte eine Leitfähigkeit von 3 mS/cm.

Im einem weiteren Schritt wurde eine Ionenaustauschchromatographie an DEAE-Fractogel (MERCK) vorgenommen. Dazu wurden 1000 ml der entsalzten Enzymlösung mit 1000 ml Puffer A (10 mM Tris/HCl pH 7,6, 10 mM Ca-Acetat) versetzt und in mehreren Ansätzen auf eine DEAE-Fractogel-Säule (Höhe 235 mm, Durchmesser 50 mm) aufgetragen. Die Säule wurde mit Puffer A gespült. Die Elution erfolgte in einem kontinuierlichen Gradienten von Puffer A zu Puffer B (Puffer A + 1 M NaCl). Es wurde bei einer Elutionsgeschwindigkeit von 4 ml 1 min eluiert und Fraktionen zu je 20 ml aufgefangen.

Die Fraktionen wurden auf Anwesenheit der LAP getestet. Dies geschah durch Messung der LAP-Aktivität. Eine LAP-Einheit ist danach definiert als die Enzymmenge, die in einer 0,0016 molaren wäßrigen Lösung von L-Leucin-p-nitroanilid bei pH 7,0 und 30° C eine Hydrolysegeschwindigkeit von 1 Mikromol pro Minute bewirkt.

Die Messung der LAP-Aktivität wurde wie folgt ausgeführt. 0,3 ml Emzymlösung wurden zu 4 ml bei 30° C vortemperierter Substratlösung (0,0016 m L-Leucin-p-nitroanilid: 0,021 g L-Leucin-p-nitroanilid (Novabiochem Best.-Nr. 03-32-0045) wurden in 2 ml Dimethylsulfoxid p.a. (Merck) gelöst und mit 0,05 m Tris/HCl pH 7,0 auf 50 ml aufgefüllt) in einer 1 cm dicken Meßküvette gegeben.

Die Extinktion bei 405 nm wurde nach 10 min bei 30 Grad Celsius im Vergleich zur Substratlösung ohne Enzym als ΔE_{405nm} im Photometer bestimmt. Die Enzymlösung wurde dazu mit 0,05 m Tris/HCl pH 7,0 so verdünnt, daß Extinktionswerte im Bereich von 0,3 bis 0,7 erhalten wurden. Der Gehalt an LAP wurde nach folgender Formel errechnet.

$$LAP - Units = \frac{\Delta E_{405nm} \cdot Analysenvolumen\ (ml) \cdot 10^6}{9620 \cdot Volumen\ Enzymlösung\ (ml) \cdot Enzymkonz. \cdot t_{min} \cdot d}$$

9620 = molarer Extinktionskoeffizient für Leucin-p-Nitroanilid

Analysenvolumen = 4,3 ml
Volumen Enzymlösung = 0,3 ml
Enzymkonzentration = Verdünnungsfaktor der Enzymlösung
t_{min} = 10 min
d (Schichtdicke der Meßküvette) = 1 cm

Die Elution der LAP setzte bei ca. 0,1 M NaCl ein. Die LAP-haltigen Fraktionen aus mehreren Läufen wurden vereinigt, gegen H_2O dest. dialysiert und anschließend lyophilisiert. Das Lyophilisat wurde in 90 ml Puffer A aufgenommen. Im nächsten Schritt wurden die Proben in mehreren Ansätzen auf eine Mono Q (Pharmacia) — Anionenaustausch-Chromatographie-Säule aufgetragen und wiederum in einem kontinuierlichem Gradienten von Puffer A zu Puffer B eluiert. Die Hauptmenge an LPA eluierte dabei zwischen 120 und 180 ml NaCL Das Eluat, 1888 ml, wurde gegen H_2O dest. dialysiert und anschließend lyophilisiert.

Das Lyophilisat wurde in 52 ml Puffer A aufgenommen und in 4 Läufen a 13 ml über eine Sephacryl S-200 HR (Pharmacia) — Säule durch Gelchromatographie weiter gereinigt. Die LAP-haltigen Fraktionen wurden vereinigt. Die spezifische Aktivität der gereinigten Fraktionen lag bei ca. 55.000 LAP Units/mg Protein. Das gereinigte Protein zeigt in der SDS-Gelelekrophorese eine einheitliche Bande mit einem Molekulargewicht von ca. 37.000 Dalton. Der isoelektrische Punkt liegt bei ca. pH 4,4. Das auf diese Weise gereinigte Protein wurde zur Sequenzierung verwendet.

Beispiel 2

Sequenzierung der LAP aus A. sojae

Die N-terminale Aminosäure-Sequenz des gereinigten Proteins wurde unter Verwendung eines Gasphasensequenators (Applied Biosystems Model 470A) bestimmt. Die bestimmte Sequenz lautete:
N-Tyr-Pro-Asp-Ser-Val-Gln-His-Xaa-Glu-Thr-Val-Gln-Asn-Leu-Ile-Asn-Ser-Leu-Asp-Lys-Lys-Asn-Phe-Glu-Thr-Val-Leu-Gln-Pro-Ile-Ser-Glu-Phe-His-Asn-Arg-COOH.

Weiterhin wurde wurde das gereinigte Enzym mittels Trypsin gespalten und die erhaltenen Peptide mittels Gelchromatographie getrennt und ebenfalls sequenziert. Eines dieser tryptischen Peptide wurde als T15 bezeichnet und diente später für die Ableitung eines Oligonukleotids. Die von T15 erhaltene Aminosäuresequenz lautete:
N-Thr-Ile-Val-Leu-Gly-Ala-His-Gln-Asp-Ser-Ile-Asn-Leu-Asn-COOH.

Beispiel 3

Ableitung und Synthese einer DNA-Sonde

Aus der N-terminalen Aminosäuresequenz wurde eine Teilsequenz für die Ableitung der Oligonukleotid-DNA-Sonde KD5 zugrunde gelegt, wobei die aus EP 388 593 bekannte Codon-Usage des Pektinesterase-Gens aus A. niger genutzt wurde. Dabei wurde das 5'-Ende so geändert, daß eine SalI-Restriktionsenzymschnittstelle erhalten wurde. Die Teilsequenz aus der N-terminalen Aminosäuresequenz lautet:
N-Thr-Val-Leu-Gln-Pro-Ile-Ser-Glu-Phe-His-Asn-COOH.
Die Sequenz der abgeleiteten DNA-Sonde KD5 lautet (Die Position der SalI-Schnittstelle ist unterstrichen):
5'-CGC GTCGACA GTA CTT CAG CCC ATC TCG GAG TTC CAAAA-3'.
Eine Teilsequenz aus dem tryptischen Peptid T15 diente als Vorlage für die DNA-Sonde KD4. Die Teilsequenz lautet:
N-Leu-Gly-Ala-His-Gln-Asp-Ser-Ile-Asn-COOH.
Die Sequenz der DNA-Sonde KD4 lautet:
5'-CTC GGC GCG CAC CAG GAC TCC ATC AA-3'.
Die Synthese der DNA-Sonden erfolgte nach dem Phosphoramiditverfahren nach Beaucage, S. L. und Caruthers, M. H. (1981) Tetrahedron Letters 22, S. 1859—1862, mittels eines DNA-Synthesizers (Applied Biosystems 380 A).

Beispiel 4

Herstellung von induziertem Zellmaterial zur Isolierung von RNA

Ausgehend von zwei Petrischalen mit 14 Tage alten Kulturen von A. sojae RH3782 auf Kartoffel-Glucose-Agar (MERCK) wurde eine Sporensuspension in ca. 20 ml destilliertem Wasser mit 0,85% NaCL und 0,1% Tween® hergestellt. Erlenmeyerkolben mit 1 Liter Volumen und zwei Schikanen, befüllt 100 ml LAP-Medium wurden mit mit je 1 ml Sporensuspension beimpft. Die Kolben wurden für 120 Stunden bei 28° Celsius unter Schütteln bei 150 Umdrehungen pro Minute inkubiert.

LAP-Medium:
4% Maisschrot
2% K_2HPO_4
2% Milchhefe (Otto Aldag)
1,4% KH_2PO_4

0,12% $MgCl_2 \times 6\,H_2O$
0,05% $CaCl_2$

Der pH-Wert wird auf 7,0 eingestellt. Die Sterilisation erfolgt 15 Minuten bei 121°C im Autoklaven.

Anschließend wurde das Mycel durch Filtration über sterile Nylonfilter abgetrennt und kurz zwischen Papiertüchern mit leichtem Druck gepreßt,um überschüssiges Medium zu entfernen. Das Mycel aus je zwei bis vier Schüttelkolben wurde dann portionsweise in kleine Plastikbeutel gefüllt, die sofort in flüssigen Stickstoff überführt wurden. Das tiefgefrorene Mycel wurde bis zur Weiterverwendung bei −80°C gelagert. Der Kulturüberstand wurde auf LAP-Aktivität analysiert und enthielt 686 LAP Units/g.

Beispiel 5

Präparation von RNA aus den induzierten Zellen aus Beispiel 5

Für die Isolierung der RNA wurde die Methode nach Vierula P. J. und Kapoor, M. (1989) J. Biol. Chem. 264, S. 1108—1114 verwendet.

Beispiel 6

Herstellung von Zellmaterial zur Isolierung von DNA

Die Herstellung von Zellmaterial zur Präparation von DNA erfolgte analog zum Beispiel 5 mit dem Unterschied, daß statt LAP-Medium Sabouraud-Boullion (MERCK) als Medium verwendet wurde. In diesem Medium wurde ein dichteres Wachstum erhalten (Der Kulturüberstand enthielt 208 LAP-Units).

Beispiel 7

Synthese von cDNA und Isolierung eines LAP-spezifischen cDNA-Klons

Die Synthese von cDNA wurde die Methode von Russo et al. (1991) EMBO J. 10, S. 563—571, verwendet. Das Prinzip des Verfahrens beruht darauf, mittels eines Oligo(dT)-Primers, EA13, die polyadenylierte mRNA in einzelsträngige cDNA umzuschreiben. An diese cDNA-Synthese schließt sich eine Polymerase-Chain-Reaktion (PCR) an. Diese wurde mittels der genspezifischen Oligonukleotid-DNA-Sonde KD5 gestartet. Die Rückreaktion erfolgt mittels des Primers EA14, der eine Teilsequenz des Oligo(dT)-Primers EA13 aufweist. Die Sequenzen von EA13 bzw. EA14 lauten (Restriktionsschnittstellen für die Enzyme XhoI und SalI enthaltende Bereiche sind unterstrichen):

EA13: 5′-GAC TCG AGT CGA CAT CGA$(T)_{21}$A/C/G-3′
EA14: 5′-GAC TCG AGT CGA CAT CGA TT-3.

Bedingungen für die Synthese der einzelsträngigen cDNA

10 µg RNA wurden in einem Reaktionsvolumen von 50 µl in 50 mM Tris/HCl pH 8,3, 5 mM $MgCl_2$, 75 mM KCl, 5 mM DTT, 0,4 mM Desoxy-Nukleosidtriphosphate (0,4 mM dATP, 0,4 mM dCTP, 0,4 mM dGTP und 0,4 mM dTTP), mit 30 pmol EA13 und 200 Units M-MLV Reverse Transkriptase (Gibco-BRL) versetzt. Die Inkubationsdauer betrug 45 min bei 42°C.

Versuchsbedingungen für die PCR-Reaktion

Das Reaktionsvolumen betrug 100 µl. Die Reaktion wurde in 20 mM Tris/HCl pH 8,4, 50 mM KCl, 1,5 mM $MgCl_2$, 0,2 mM Desoxy-Nukleosidtriphosphate (0,2 mM dATP, 0,2 mM dCTP, 0,2 mM dGTP und 0,2 mM dTTP), mit 70 pmol KD5 und 5 Units Taq-DNA-Polymerase ausgeführt. Der Ansatz wurde für 20 PCR-Zyklen (94°C, 40 sec — 50°C, 2 min — 72°C, 3 min pro Zyklus) inkubiert.

Anschließend wurden die PCR-Produkte gelelektrophoretisch aufgetrennt. Es wurde im Bereich von 1 kb eine Bande erhalten, die mit der Oligonukleotidsonde KD4 hybridisierte. Der PCR-Ansatz wurde mit Ethanol gefällt, mit SalI geschnitten und mit Sal geschnittenes Plasmid pUC18 ligiert. Die Plasmide wurden in E. coli DH5α transformiert. Nach dem Plattieren des Reaktionsansatzes wurden 131 weiße Kolonien erhalten (im Gegensatz zu blauen Kolonien mit Plasmiden ohne Insertionen), von denen mittels Koloniehybridisierung mit der radioaktiv markierten Oligonukleotidsonde KD4 der Klon T128 ausgewählt wurde, der ein Plasmid mit einer Insertion von 1 kb aufwies. Dieses Plasmid wurde als pKD4 bezeichnet. Die Insertion wurde sequenziert. Die von der DNA abgeleitete Aminosäuresequenz enthielt, wie erwartet, die bei der Proteinsequenzierung der LAP gefundenen Peptidsequenzen. Die Sequenz ist unter SEQ ID NO. 1 aufgeführt.

Beispiel 8

Isolierung des chromosomalen LAP-Gens

Chromosomale DNA aus A. sojae wurde mittels Phenolextraktion aus dem Zellmaterial aus Beispiel 7 isoliert und partiell mit dem Restriktionsenzym Sau3A gespalten. Die DNA wurde mittels einer Saccharose-Gradien-

ten-Zentrifugation nach Größe fraktioniert. DNA aus Fraktionen mit Fragmenten, die größer als 10 kb waren wurden in den Phagen Lambda EMBL3 kloniert. Es wurden ca. 250 000 rekombinante Phagen erhalten, die auf großen Agar-Platten (Nunc-Schalen) mit je ca. 15 000 Plaque bildenden Einheiten pro Platte verteilt wurden. Von den Platten wurde die DNA der Phagen auf entsprechende Nitrocellulose-Filter für die Hybridisierung übertragen. Als Probe zu Detektierung von Phagen, die das chromosomale LAP-Gen enthalten wurde das SalI cDNA-Fragment aus dem Plasmid pKD4 (siehe Beispiel 8) verwendet. Es wurde isoliert und radioaktiv markiert. Die Nitrocellulose-Filter wurden mit der markierten Probe für 18 h bei 65° C hybridisiert. Anschließend wurden die Filter bei 65° C mit zweifach SSC-Puffer mit 0,1% SDS gewaschen. Mittels Autoradiographie wurden zwei positive Klone detektiert, von den Platten mit den rekombinanten Phagen isoliert. Die Klone wurden nochmals plattiert und im gleichen Verfahren rehybridisiert. Von einem der Klone erfolgte eine Subklonierung eines hybridisierenden, 1,8 kb großen Hind/III/BamHI-Fragmentes in das Plasmid pUC18. Das erhaltene Plasmid wurde als pKD12 bezeichnet.

Die Nukleotidsequenz der 1,8 kb Insertion wurde bestimmt und ist in SEQ ID NO: 1 wiedergegeben. Durch den Vergleich mit der cDNA-Sequenz und mit der Sequenz wurde das Vorhandensein und die Lage des chromosomalen LAP-Gens ermittelt. Vor dem ATG-Startcodon des LAP-Gens befindet sich ein 581 Nukleotide langer Upstream-Bereich, der als Promotor funktionell ist. Das LAP-Gen weist eine Signalpeptidsequenz von 47 Aminosäuren auf. Das Strukturgen enthält zwei Introns. Hinter dem Stopcodon befinden sich 129 Nukleotide, die als Terminator fungieren können.

Beispiel 9

Transformationsmethode für Aspergillus und Trichoderma reesei-Stämme

Von einer ca. zwei Wochen alten Petrischalenkultur des zu transformierenden Pilzstammes wurde eine Sporensuspension in ca. 10 ml 0,85% NaCl durch Abschwemmen unter Zuhilfenahme eines Spatels hergestellt. Es wurden je vier 1 l Schüttelkolben mit 100 ml Czapek-Dox-Minimalmedium (Oxoid) mit 0,1% Hefeextrakt mit je 1 ml Sporensuspension beimpft und ca. 16 Stunden bei 28° C auf einem Rundschüttler bei 120 Umdrehungen pro Minute inkubiert. Das Mycel aus je vier Schüttelkolben wurde über einem Papierfilter geerntet und mit ca. 50 ml MP-Puffer (1,2 $MgSO_4$ in 10 mM Phosphatpuffer pH 5,8) gespült. Nach dem Ablaufen des Puffers wurde das feuchte Mycel gewogen. In der Regel wurden ca. 3 bis 5 g Feuchtmycel erhalten.

Pro g Feuchtmycel wurden 5 ml MP-Puffer, 120 µl Novozym-Lösung (1 g Novozym® 234 (Novo Nordisk) in 6 ml MP-Puffer), und 25 µl β-Glucuronidase (Sigma) zugegeben. Die Mycel-Suspension wurde wurde 5 min in Eiswasser gestellt. Anschließend wurden 60 µl Rinderserumalbumin-Lösung (0,2 g Rinderserumalbumin in 4 ml MP-Puffer, sterilfiltriert) zugegeben und der Ansatz unter leichtem Schütteln bei 30° C inkubiert. Die Bildung von Protoplasten wurde visuell im Mikroskop verfolgt. Wenn keine wesentliche Zunahme der Protoplastenbildung mehr festgestellt wurde, wurde der Ansatz zur Ernte der Protoplasten abgebrochen. Dies war in der Regel nach etwa 3 bis 5 Stunden der Fall.

Die Protoplastensuspension wurde zur Abtrennung noch vorhandener grober Mycelbestandteile über ein mit MP-Puffer getränktes Glaswollefilter gegeben und in Zentrifugenröhrchen überführt. Die obere Hälfte der Röhrchen wurde mit 600 mM Sorbitol, 100 mM Tris/HCl pH 7,0 überschichtet. Die Röhrchen wurden 10 min bei 2500 g zentrifugiert. Die Protoplasten wurden aus der Zwischenschicht abgenommen und in 1 M Sorbitol, 10 mM Tris/HCl pH 7,5 aufgenommen. Anschließend wurden die Protoplasten zweimal mit STC-Puffer (1 M Sorbitol, 10 mM Tris/HCl pH 7,5, 10 mM $CaCl_2$) durch Zentrifugation bei 1500 g gewaschen und zuletzt in 1 ml STC-Puffer aufgenommen.

Zur Transformation von A. oryzae oder A. sojae wurden 300 µl Protoplastensuspension ca. 10 µg p3SR2 als Selektionsplasmid und 10 µg des jeweiligen Plasmids zur Expression der LAP in 25 µl 10 mM Tris/HCl pH 8,0 zusammengegeben und 10 min bei 0° C inkubiert. Anschließend wurde nochmals 25 µl der gleichen DNA-Menge und 400 µl PEG-Lösung (60% Polyethylenglykol 6000 (Fluka) in 10 mM Tris/HCl pH 7,5, 50 mM $CaCl_2$) zusammengegeben, sehr vorsichtig vermischt und 5 min bei Raumtemperatur inkubiert. Es wurden nochmals 600 µl PEG-Lösung zugegeben, vermischt und der Ansatz für weitere 20 min bei Raumtemperatur inkubiert. Zu dem Ansatz wurde mit ca. 9 ml Acetamid-Weichagar (Minimalmedium mit 10 mM Acetamid als einziger N-Quelle, 1 M Saccharose, 0,6 Gew.-% Agar) bei 45° C gemischt und auf vier Petrischalen mit dem gleichen Medium, jedoch mit 1,5 Gew.-% Agar (Oxoid) und zusätzlich 15 mM CsCl, verteilt. Die Platten wurden bei 28° C inkubiert. Nach 6 bis 10 Tagen wurden schnell wachsende Kolonien (Transformanten) auf Acetamid-Medium ohne Saccharose isoliert, zweimal über Einzelsporkolonien gereinigt und zuletzt auf Vollmedium, z. B. Kartoffel-Dextrose-Agar übertragen.

Die Transformation von Stämmen der Arten A. niger, A. awamori, A. foetidus, A. japonicus oder A. phoenicis kann ebenfalls mit dem Plasmid p3SR2 erfolgen. Bevorzugt wurde die Transformation jedoch mit dem Plasmid pAN7-1 ausgeführt. Die Protoplastenpräparation und die Zugabe von Plasmid-DNA erfolgt in analoger Weise wie oben für das Plasmid p3SR2 beschrieben. Statt der Zugabe von Acetamid-Weichagar wird jedoch der gesamte Transformationsansatz zu 100 ml Czapek-Dox-Minimalmedium (Oxoid) mit 100 µg Hygromycin B/ml, 1,5 Gew.-% Agar (Oxoid) und 1 M Saccharose, abgekühlt auf ca. 45 Grad Celsius gegeben und vorsichtig vermengt. Der Ansatz wird dann in Protionen zu je 10 ml in Petrischalen gegeben in denen jeweils 10 ml Czapek-Dox-Minimalmedium (Oxoid) mit 1,5 Gew.-% Agar (Oxoid), jedoch ohne Hygromycin und ohne Saccharose, als feste Unterschicht vorgelegt war. Nach dem Erstarren der oberen Agarschicht werden die Petrischalen bei 37 Grad Celsius inkubiert. Gegen Hygromycin B resistente Transformanten können nach ca. 3—10 Tagen abgeimpft werden und zur Überprüfung der Resistenz auf Czapek-Dox-Minimalmedium (Oxoid) mit 50 µg Hygromycin B/ml und 1,5 Gew.-% Agar (Oxoid) übertragen werden.

Beispiel 10

Herstellung einer LAP sekretierenden Transformante

Der Stamm A. awamori NRRL 3112 wurde gemäß Beispiel 9 mit pAN7-1 und pKD12 cotransformiert. Es wurden eine Vielzahl Hygromycin B resistenter Transformanten erhalten und in Schüttelkolben unter Verwendung des im Beispiel 4 angegebenen LAP-Mediums auf Produktion von LAP geprüft. Die Kulturüberstände der Transformante A. awamori RH 3827 enthielten in mehreren Versuchen ca. 5000—10 000 LAP-Units/mL Der Stamm wurde bei der DSM hinterlegt.

Rekombinant hergestellte Leucinaminopeptidase aus Aspergillus sojae

Sequenzprotokoll

(1) ALLGEMEINE ANGABEN:

(i) ANMELDER:
(A) NAME: Roehm GmbH
(B) STRASSE: Kirschenallee
(C) ORT: Darmstadt
(E) LAND: Germany
(F) POSTLEITZAHL: D-64293
(G) TELEFON: (06151)184102
(H) TELEFAX: (06151)184178
(I) TELEX: 419 474-0 rd d

(ii) BEZEICHNUNG DER ERFINDUNG: Leucin-Aminopeptidase aus A. sojae

(iii) ANZAHL DER SEQUENZEN: 2

(iv) COMPUTER-LESBARE FASSUNG:
(A) DATENTRGER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) BETRIEBSSYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPA)

(2) ANGABEN ZU SEQ ID NO: 1:

(i) SEQUENZKENNZEICHEN:
(A) LNGE: 1873 Basenpaare
(B) ART: Nucleotid
(C) STRANGFORM: Doppelstrang
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKLS: Genom-DNA

(iii) HYPOTHETISCH: NEIN

(iv) ANTISENSE: NEIN

(ix) MERKMAL:
(A) NAME/SCHLSSEL: exon
(B) LAGE:582..1064

(ix) MERKMAL:
(A) NAME/SCHLSSEL: intron
(B) LAGE:1065..1132

(ix) MERKMAL:
(A) NAME/SCHLSSEL: exon
(B) LAGE:1133..1450

(ix) MERKMAL:
(A) NAME/SCHLSSEL: intron
(B) LAGE:1451..1507

(ix) MERKMAL:
(A) NAME/SCHLSSEL: exon
(B) LAGE:1508..1741

(ix) MERKMAL:
(A) NAME/SCHLSSEL: CDS
(B) LAGE:join(582..1064, 1133..1450, 1508..1744)

(ix) MERKMAL:
(A) NAME/SCHLSSEL: sig_peptide
(B) LAGE:582..722

(ix) MERKMAL:
(A) NAME/SCHLSSEL: mat_peptide
(B) LAGE:723..1741

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 1:

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AAGCTTGGCA GGGCGGGTTC CCTACGGACC CCCGGCGGGG AATCCCTGAT GTATCCATGC      60

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GATCCCATGA CTTCAGGTTA CAATGGTCTC TGCAGATACA GCTTACTTCC CGGCATTATA     180

GGAAGGTCGG AGAGGTGCCA AGTGTTGCCA AGCAGCTCCT CATATCTTGC ATCATACTTG     240

GCCCGGAATA TTCTATGGCG TCAAATGTAA TACACCGTAC CTTGCCATGT GACTAATTCG     300

CTGGTCTATA AAACCTCGAG TTCTGTCTTT CCATAAAGTG GATCGTTCCC TCATAATCAG     360

TCTCCCTCGG CTACTCGAGC ATTCCGTTCG TATCGACTTG GTGATACACG CTTTTGTTCG     420

CTCAATATGC GTTTCCTCCC CTGCATCGCG ACCTTGGCAG CCACGGCCTC TGCCCTTGCT     480

ATTGGAGACC ATATCCGTTC GGACGATCAG TATGTCCTAG AACTTGGCCC GGGAGAAACG     540

AAAGTTGTTA CGGAAGCAGA GAAATGGGCT CTGAGGGCTG T ATG ACG AAA TTT         593
                                               Met Thr Lys Phe
                                               -47     -45

CCC TTT GAC GTT TAC GCC CAG CAT CCC GCT AAT AAG ATT GAA CAG GAG       641
Pro Phe Asp Val Tyr Ala Gln His Pro Ala Asn Lys Ile Glu Gln Glu
          -40                 -35                 -30

GGC AAG CGT TTT TTC GAT ATA ACT GGA CGG ACC AGT AGC CTG GAA CTC       689
Gly Lys Arg Phe Phe Asp Ile Thr Gly Arg Thr Ser Ser Leu Glu Leu
     -25                 -20                 -15

GCA TCG AAC AAG AAG CAA AAA CTC GCG GTC ACC TAT CCC GAT TCC GTG       737
Ala Ser Asn Lys Lys Gln Lys Leu Ala Val Thr Tyr Pro Asp Ser Val
-10                  -5                   1                   5

CAA CAT AAC GAG ACC GTG CAA AAC CTA ATC AAC TCG CTC GAC AAA AAG       785
Gln His Asn Glu Thr Val Gln Asn Leu Ile Asn Ser Leu Asp Lys Lys
            10                  15                  20

AAC TTT GAA ACC GTT CTC CAG CCG TTC TCG GAG TTC CAC AAT CGC TAT       833
Asn Phe Glu Thr Val Leu Gln Pro Phe Ser Glu Phe His Asn Arg Tyr
        25                  30                  35

TAT AAG AGC GAC AAT GGT AAG AAA TCA TCC GAG TGG CTG CAA GGC AAG       881
Tyr Lys Ser Asp Asn Gly Lys Lys Ser Ser Glu Trp Leu Gln Gly Lys
     40                  45                  50

ATT CAG GAA ATC ATC TCC GCC AGT GGA GCA AAG GGA GTC ACT GTG GAG       929
Ile Gln Glu Ile Ile Ser Ala Ser Gly Ala Lys Gly Val Thr Val Glu
 55                  60                  65

CCT TTC AAA CAC TCC TTC CCG CAG TCG AGT TTG ATT GCG AAG ATC CCC       977
Pro Phe Lys His Ser Phe Pro Gln Ser Ser Leu Ile Ala Lys Ile Pro
70                  75                  80                  85
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GGC AAG AGT GAC AAA ACC ATC GTT CTT GGA GCG CAT CAG GAC TCC ATC                1025
Gly Lys Ser Asp Lys Thr Ile Val Leu Gly Ala His Gln Asp Ser Ile
                 90                  95                 100

AAC CTC AAT TCG CCT TCA GAG GGC CGT GCA CCA GGT GCT GGTGGGTACT                1074
Asn Leu Asn Ser Pro Ser Glu Gly Arg Ala Pro Gly Ala
            105                 110

TCGCACGTCC TGTCCATGAA CCATAGAACA TCGTGATGCT AACAGAGACG CGTGGTTA               1132

GAT GAC GAT GGA TCC GGT GTT GTT ACC ATC CTT GAA GCC TTC CGC GTT               1180
Asp Asp Asp Gly Ser Gly Val Val Thr Ile Leu Glu Ala Phe Arg Val
115                 120                 125                 130

CTC CTG ACG GAC GAG AAG GTT GCG GCC GGT GAG GCT CCG AAC ACC GTT               1228
Leu Leu Thr Asp Glu Lys Val Ala Ala Gly Glu Ala Pro Asn Thr Val
                135                 140                 145

GAG TTC CAC TTC TAT GCC GGA GAG GAG GGA GGT CTT CTG GGA AGT CAG               1276
Glu Phe His Phe Tyr Ala Gly Glu Glu Gly Gly Leu Leu Gly Ser Gln
            150                 155                 160

GAT ATC TTT GAG CAG TAC TCC CAG AAA AGC CGA GAT GTG AAA GCC ATG               1324
Asp Ile Phe Glu Gln Tyr Ser Gln Lys Ser Arg Asp Val Lys Ala Met
        165                 170                 175

CTC CAG CAG GAT ATG ACG GGT TAT ACC AAA GGC ACC ACT GAT GCT GGA               1372
Leu Gln Gln Asp Met Thr Gly Tyr Thr Lys Gly Thr Thr Asp Ala Gly
    180                 185                 190

AAG CCA GAG TCG ATC GGC ATC ATC ACC GAC AAT GTC GAT GAG AAC CTG               1420
Lys Pro Glu Ser Ile Gly Ile Ile Thr Asp Asn Val Asp Glu Asn Leu
195                 200                 205                 210

ACC AAG TTC CTG AAG GTC ATT GTC GAT GCT GTAAGTTTCA AAACCTGTTT                 1470
Thr Lys Phe Leu Lys Val Ile Val Asp Ala
                215                 220

GTGGTAGTCC CTTCATGCTT ACACTGGATA CTTGTAG TAT TGC ACT ATC CCG ACC              1525
                                        Tyr Cys Thr Ile Pro Thr
                                                        225

GTC GAT TCG AAA TGC GGA TAC GGA TGC TCT GAC CAT GCT TCT GCC ACG               1573
Val Asp Ser Lys Cys Gly Tyr Gly Cys Ser Asp His Ala Ser Ala Thr
            230                 235                 240

AAG TAT GGT TAT CCC GCC GCA TTT GCA TTC GAG TCA GCC TTT GGC GAC               1621
Lys Tyr Gly Tyr Pro Ala Ala Phe Ala Phe Glu Ser Ala Phe Gly Asp
        245                 250                 255

GAC AGC CCT TAC ATT CAC TCG GCC GAT GAT ACG ATT GAG ACC GTC AAC               1669
Asp Ser Pro Tyr Ile His Ser Ala Asp Asp Thr Ile Glu Thr Val Asn
    260                 265                 270

TTT GAC CAT GTG CTG CAA CAC GGC AGA CTG ACT CTT GGA TTT GCA TAT               1717
Phe Asp His Val Leu Gln His Gly Arg Leu Thr Leu Gly Phe Ala Tyr
275                 280                 285                 290

GAG CTT GCC TTC GCA GAT TCA CTG TAA GGCTTATGAT GACGGTTGTA                     1764
Glu Leu Ala Phe Ala Asp Ser Leu  *
                295

TGAGCGAGAG ATCCAGTCCA ACAGTGTGTA TAATATGTGG GCCTGTGTTC AAATAGCACT             1824

TTGATTTAGC CCGCGATTAG CTTTCGTGAC GAAAATAGAG GCCGAATTC                         1873
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(2) ANGABEN ZU SEQ ID NO: 2:

(i) SEQUENZKENNZEICHEN:
(A) LNGE: 346 Aminosuren
(B) ART: Aminosure
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKLS: Protein
(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 2:

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Met Thr Lys Phe Pro Phe Asp Val Tyr Ala Gln His Pro Ala Asn Lys
-47  -45                 -40                 -35

Ile Glu Gln Glu Gly Lys Arg Phe Phe Asp Ile Thr Gly Arg Thr Ser
 -30                 -25                 -20

Ser Leu Glu Leu Ala Ser Asn Lys Lys Gln Lys Leu Ala Val Thr Tyr
-15                 -10                  -5                   1

Pro Asp Ser Val Gln His Asn Glu Thr Val Gln Asn Leu Ile Asn Ser
             5                  10                  15

Leu Asp Lys Lys Asn Phe Glu Thr Val Leu Gln Pro Phe Ser Glu Phe
         20                  25                  30

His Asn Arg Tyr Tyr Lys Ser Asp Asn Gly Lys Lys Ser Ser Glu Trp
     35                  40                  45

Leu Gln Gly Lys Ile Gln Glu Ile Ile Ser Ala Ser Gly Ala Lys Gly
 50                  55                  60                  65

Val Thr Val Glu Pro Phe Lys His Ser Phe Pro Gln Ser Ser Leu Ile
                 70                  75                  80

Ala Lys Ile Pro Gly Lys Ser Asp Lys Thr Ile Val Leu Gly Ala His
             85                  90                  95

Gln Asp Ser Ile Asn Leu Asn Ser Pro Ser Glu Gly Arg Ala Pro Gly
        100                 105                 110

Ala Asp Asp Asp Gly Ser Gly Val Val Thr Ile Leu Glu Ala Phe Arg
    115                 120                 125

Val Leu Leu Thr Asp Glu Lys Val Ala Ala Gly Glu Ala Pro Asn Thr
130                 135                 140                 145

Val Glu Phe His Phe Tyr Ala Gly Glu Glu Gly Gly Leu Leu Gly Ser
                150                 155                 160

Gln Asp Ile Phe Glu Gln Tyr Ser Gln Lys Ser Arg Asp Val Lys Ala
            165                 170                 175

Met Leu Gln Gln Asp Met Thr Gly Tyr Thr Lys Gly Thr Thr Asp Ala
        180                 185                 190

Gly Lys Pro Glu Ser Ile Gly Ile Ile Thr Asp Asn Val Asp Glu Asn
    195                 200                 205

Leu Thr Lys Phe Leu Lys Val Ile Val Asp Ala Tyr Cys Thr Ile Pro
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210                 215                 220                 225

Thr Val Asp Ser Lys Cys Gly Tyr Gly Cys Ser Asp His Ala Ser Ala
                230                 235                 240

Thr Lys Tyr Gly Tyr Pro Ala Ala Phe Ala Phe Glu Ser Ala Phe Gly
            245                 250                 255

Asp Asp Ser Pro Tyr Ile His Ser Ala Asp Asp Thr Ile Glu Thr Val
        260                 265                 270

Asn Phe Asp His Val Leu Gln His Gly Arg Leu Thr Leu Gly Phe Ala
    275                 280                 285

Tyr Glu Leu Ala Phe Ala Asp Ser Leu  *
290                 295
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Patentansprüche

1. Eine rekombinante Desoxyribonukleinsäure (DNA) isolierbar aus Aspergillus sojae, **dadurch gekennzeichnet,** daß sie für eine Leucin-Aminopeptidase (LAP) codiert und eine Nukleotidsequenz entsprechend der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz oder eine davon abgeleitete Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, aufweist.

2. Ein Vektor enthaltend
   a) DNA-Sequenzen zur Replikation des Vektors in E. coli
   b) DNA-Sequenzen zur Expression und Sekretion eines Polypeptids in einem Aspergillus-Stamm oder in einem Trichoderma reesei-Stamm, die für einen Promotor, eine Signalpeptidsequenz und optional für einen Terminator codieren
   c) eine für ein Polypeptid codierende DNA-Sequenz, die funktionell mit den DNA-Sequenzen nach b) verbunden ist,

dadurch gekennzeichnet, daß die DNA-Sequenz nach c) eine Nukleotidsequenz entsprechend der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz oder eine davon abgeleitete Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, aufweist.

3. Vektor nach Anspruch 2, dadurch gekennzeichnet, daß der Vektor ein Plasmid ist.

4. Vektor nach Anspruch 3, dadurch gekennzeichnet, daß der Vektor das Plasmid pkD12 ist.

5. Vektor nach Anspruch 2, dadurch gekennzeichnet, daß der Vektor ein Phage oder ein Cosmid ist.

6. Vektor nach Anspruch 2, dadurch gekennzeichnet, daß die DNA-Sequenzen nach b), die für einen Promotor codieren, ausgewählt sind aus der Gruppe TAKA-Amylase A-Promotor, gpdA-Promotor aus Aspergillus nidulans, Pektinesterase-Promotor aus Aspergillus niger, Polygalakturonidase-Promotor aus Aspergillus niger, Glucoamylase-Promotor aus Aspergillus niger oder Aspergillus awamori, Leucinaminopeptidase-Promotor aus Aspergillus sojae, Cellobiohydrolase(cbhI)-Promotor aus Trichoderma reesei.

7. Vektor nach Anspruch 2, dadurch gekennzeichnet, daß die DNA-Sequenzen nach b), die für eine Signalpeptidsequenz codieren, die ausgewählt aus der Gruppe TAKA-Amylase A-Signalpeptidsequenz, Pektinesterase-Signalpeptidsequenz aus Aspergillus niger, Polygalakturonidase-Signalpeptidsequenz aus Aspergillus niger, Glucoamylase-Signalpeptidsequenz aus Aspergillus niger oder Aspergillus awamori, Leucinaminopeptidase-Signalpeptidsequenz aus Aspergillus sojae. Cellobiohydrolase Signalpeptidsequenz (cbhI) aus Trichoderma reesei.

8. Transformierter Wirtsorganismus geeignet zur Herstellung von Leucinaminopeptidase, dadurch gekennzeichnet, daß der Wirtsorganismus ein Aspergillus-Stamm oder ein Trichoderma reesei-Stamm ist und mit einem Vektor nach Anspruch 2 transformiert ist.

9. Transformierter Wirtsorganismus nach Anspruch 8, dadurch gekennzeichnet, daß der Wirtsorganismus ein Stamm von Aspergillus niger, Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus phoenicis, Aspergillus oryzae, Aspergillus sojae oder Trichoderma reesei ist.

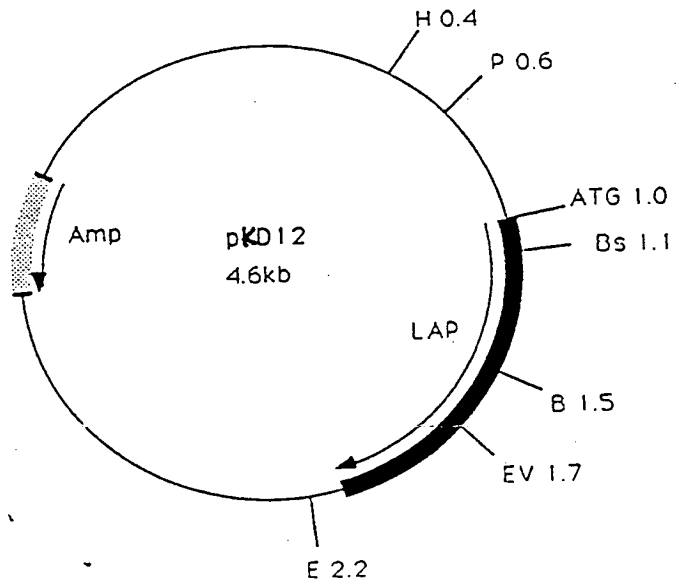
10. Transformierter Wirtsorganismus nach Anspruch 9, dadurch gekennzeichnet, daß der Wirtsorganismus Aspergillus sojae RH3782 ist.

11. Verfahren zur Herstellung von Leucinaminopeptidase durch Fermantation eines transformierten Wirtsorganismus in einem geeigneten zellfreien Kulturfiltrat, dadurch gekennzeichnet, daß der transformierte Wirtsorganismus ein Aspergillus-Stamm oder ein Trichoderma reesei Stamm ist und mit einem Vektor gemäß Anspruch 2 transformiert ist.

12. Verfahren nach Anspruch 11, dadurch gekennzeichnet, daß der transformierte Wirtsorganismus ein Stamm von Aspergillus niger, Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus phoenicis, Aspergillus oryzae, Aspergillus sojae oder Trichoderma reesei ist.

13. Verfahren nach Anspruch 11 oder 12, dadurch gekennzeichnet, daß der transformierte Wirtsorganismus eine Transformante von Aspergillus sojae RH3782 ist.

Nummer: DE 195 26 485 A1
Int. Cl.[6]: C 12 N 15/80
Offenlegungstag: 23. Januar 1997



EV= EcoRV
Bs= BstEII
E. = EcoRI
H. = HindIII
P. = PstI
B. = BamHI

Fig 1: Schematische Darstellung des Vectors pKD12

14. Verfahren nach Anspruch 11, dadurch gekennzeichnet, daß der transformierte Wirtsorganismus Aspergillus awamori RH3827 ist.
15. Enzymprodukt, geeignet zur Proteinhydrolyse, dadurch gekennzeichnet, daß es eine mittels eines rekombinanten Aspergillus- oder T. reesei-Stammes hergestellte Leucinaminopeptidase enthält.
16. Enzymprodukt nach Anspruch 15, dadurch gekennzeichnet, daß sich die Leucinaminopeptidase von einer rekombinanten Desoxyribonukleinsäure (DNA) isolierbar aus Aspergillus sojae, entsprechend der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz oder einer davon abgeleiteten Nukleotidsequenz, die unter stringenten Bedingungen mit der in SEQ ID NO: 1 für die reife LAP angegebenen Nukleotidsequenz hybridisiert, herleitet.
17. Proteinhydrolysat, dadurch gekennzeichnet, daß es mit einem Enzymprodukt nach Anspruch 15 oder 16 hergestellt wurde.

Hierzu 1 Seite(n) Zeichnungen